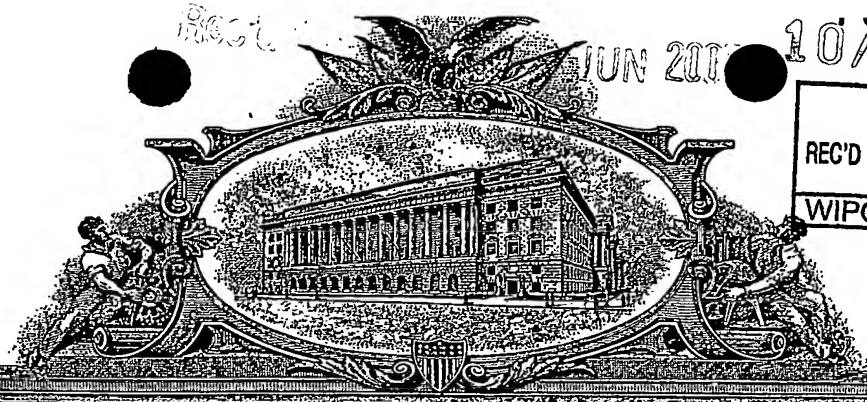


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Docket Number: SCRIP1600

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

INVENTOR(s)/APPLICANT(s)			
Given Name (first and middle (if any))	Family Name or Surname	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY)	
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<input type="checkbox"/> Additional inventors are being named on page 2 attached hereto.			
TITLE OF INVENTION (280 characters max)			
METHODS AND COMPOSITIONS ASSOCIATED WITH NOCICEPTIVE PAIN			
CORRESPONDENCE ADDRESS			
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ENCLOSED APPLICATION PARTS (check all that apply)			
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Respectfully submitted,

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for

UNITED STATES LETTERS PATENT

on

METHODS AND COMPOSITIONS ASSOCIATED
WITH NOCICEPTIVE PAIN

by

Ardem Patapoutian and Gina M. Story

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Docket No.: SCRIP1600

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METHODS AND COMPOSITIONS ASSOCIATED WITH NOCICEPTIVE PAIN

ACKNOWLEDGMENT OF GOVERNMENT SUPPORT

[0001] This invention was made in part with government support under NINDS Grant No. R01NS42822 awarded by the National Institutes of Health. The federal government may have certain rights in this invention.

FIELD OF THE INVENTION

[0002] The invention relates generally to nociceptive pain and disorders associated with pain and more specifically to polynucleotides encoding polypeptides that affect nociceptive pain and methods for use therefor.

BACKGROUND

[0003] Pain has been defined in a variety of ways. For example, pain can be defined as the perception by a subject of noxious stimuli that produces a withdrawal reaction by the subject. The most commonly experienced form of pain may be defined as the effect of a stimulus on nerve endings, which results in the transmission of impulses to the cerebrum. This somatic sensation and normal function of pain, referred to as nociception or nociceptive pain, informs the organism of impending tissue damage. Somatic and visceral free nerve endings, termed nociceptors, initially process such pain signals.

[0004] Pain is a subjective experience related to perception of inputs to the central nervous system by a specific class of sensory receptors known as nociceptors. Nociceptors fire in response to noxious thermal, mechanical and chemical stimuli. Coding of a stimulus as painful occurs at several levels in the nervous system. The first is at the level of transduction of the noxious stimulus in the peripheral nerve terminals of the nociceptors. During the transduction step, the noxious stimulus is converted to an electrical stimulus in the form of an action potential. In mammals the vanilloid receptors

(VR-1 and VRL-1) are proposed to function during transduction of a noxious heat stimulus. Candidate molecules for transducing noxious mechanical stimuli have yet to be identified.

[0005] The second level of coding occurs in the dorsal horn of the spinal cord. The cell bodies of nociceptive neurons are found in the dorsal root ganglia and send projections both to the periphery and to the dorsal horn. Upon stimulation nociceptors release the excitatory neurotransmitter glutamate, among others, which produces action potential in post-synaptic cells of the dorsal horn, which project to the brain where pain is perceived. The higher level processing involved in pain perception is poorly understood. High intensity pain is signaled through increased release of substance P by the afferent nociceptive terminals in the dorsal horn. This peptide function through the G-protein couples substance P receptor, NK-1.

[0006] In general, while brain pathways governing the perception of pain are still incompletely understood, sensory afferent synaptic connections to the spinal cord, termed "nociceptive pathways" have been studied. The nociceptive pathway, which exists for protection of the organism (such as the pain experienced in response to a burn or noxious cold), is inactive. Activity is initiated by the application of a high intensity, potentially damaging stimulus. This stimulus serves to depolarize certain classes of afferent (sensory) axons of the small unmyelinated category, designated C fibers.

[0007] The signal carried by the C fibers travels up the peripheral nerve and into the spinal cord where synapses are made on second order and higher order neurons, which then transmit the pain signal up the spinal cord in the spinothalamic tract ending in the thalamus. Polysynaptic junctions in the dorsal horn of the spinal cord are involved in the relay and modulation of sensations of pain to various regions of the brain, including the periaqueductal grey region. The ventrolateral and ventromedial thalamic nuclei project to the cortex where the pain is then processed with regard to localization and other integrative characteristics.

[0008] Analgesia, or the reduction of pain perception, can be affected directly by decreasing transmission along such nociceptive pathways. Analgesic opiates are thought to act by mimicking the effects of endorphin or enkephalin peptide-containing neurons, which synapse presynaptically at the C-fiber terminal and which, when they fire, inhibit release of substance P from the C-fiber. Descending pathways from the brain are also inhibitory to C-fiber firing. Thus, CNS-mediated analgesia leads to an overall inhibition of the pain transmission.

[0010] While neuropathic pain is known to have a number of underlying etiologies, it is characterized by a distinct set of symptoms. As described in greater detail below, these can include enhanced sensitivity to innocuous thermal-mechanical stimuli, abnormal sensitivity to noxious stimuli, tenderness, and spontaneous burning pain. Neuropathic pain is also progressive in nature, in that it generally worsens over time. Known treatment methods treat the symptoms without necessarily lessening the underlying pathology.

[0011] Typically, chronic nociceptive pain results from changes in the peripheral sensory terminal secondary to local tissue damage. Mild damage, such as abrasions or burns, and inflammation in the cutaneous receptive fields or joints will produce significant increases in the excitability of polymodal nociceptors (C fibers) and high threshold mechanoreceptors. This increased excitability leads to increased spontaneous activity and an exaggerated response to otherwise minimal stimuli.

[0012] These events have several consequences. First, the magnitude of the pain state in humans and animals is proportional to the discharge rate in such sensory afferent. The facilitated response secondary to the local peripheral injury may lead to an exaggerated pain state simply because of the increased afferent activity. Secondly, spontaneous activity in small sensory afferent causes central neurons in the spinal cord to develop an exaggerated response to subsequent input. Both of these events, secondary to the increased spontaneous activity and reactivity in small sensory afferent generated by the peripheral injury leads to a behavioral state referred to as hyperalgesia. Thus, where the pain response is the result of an exaggerated response to a given stimulus, the organism is hyperalgesic. The importance of the hyperalgesic state in the post injury pain state has

been repeatedly demonstrated and this facilitated processing appears to account for a major proportion of the post-injury/inflammatory pain state.

[0013] Despite numerous definitions, the brain pathways, mechanisms and intermediates governing the perception of pain are not completely understood. A number of analgesics and opiates are currently on the market to address the discomforts associated with pain. However, many of these agents are addictive or have side effects that often provide additional discomforts to a subject when taken over a long period of time. For example, side effects associated with a number of opiates include sedation, depression of respiration, constipation, nausea and emesis, abuse liability and the development of addiction. These effects serve to limit the utility of opiates for controlling post injury pain. Addiction liability can occur secondary to medical uses of the drug where the central effects lead to an addicted and dependent state.

[0014] The ability to sense cold as a distinct presence resides in specialized neurons within the peripheral nervous system that detect varied environmental temperature. The cell bodies of these sensory neurons reside in the vertebral column and their projections extend for long distances to peripheral tissues such as the skin. It is hypothesized that channels present at the end of these projections are activated by physical stimuli such as temperature and pressure (Hensel, Monogr Physiol Soc 38:1-321, 1981).

[0015] The cloning of TRPV1 (VR1: a capsaicin- and heat-activated channel) has proven this hypothesis and ignited research into thermosensation at the molecular level (Caterina et al., Nature 389:816-824, 1997). TRPV1 is activated near 43 °C, a temperature most mammals find noxious. Three other TRPV channels with greater than 40% amino-acid level identity to TRPV1 have since been cloned and characterized as thermosensors. These channels are activated at various heat thresholds, ranging from 33 °C (warm) for TRPV3 to 55 °C (high-threshold noxious heat) for TRPV2 (Caterina et al., Nature 398, 436-441, 1999; Peier et al., Science 296:2046-2049, 2002b; Smith et al., Nature 418:186-190, 2002; Xu et al., Nature 418:181-186, 2002). TRPV4, originally described as an osmo-sensor, has also been shown to be activated by warm temperatures. Much less is known about channels that sense cold. Recently, the cloning of a menthol-

and cold-activated channel, TRPM8 (CMR1) was described (McKemy et al., *Nature* 416:52-58, 2002; Peier et al., *Cell* 108:705-715, 2002a). The threshold of TRPM8 activation is reported to be either 28 °C or 23 °C, consistent with the pleasant/cool feeling that menthol products convey. Since TRPM8 is mostly activated at cool rather than cold temperatures, it has been postulated that other cold-activated channels exist.

[0016] The known thermoreceptors all belong to the Transient Receptor Potential (TRP) family of non-selective cation channels. TRP channels are divided into three subclasses designated TRPC, TRPV, and TRPM (Montell et al., *Mol Cell* 9:229-231, 2002). All have six putative transmembrane domains with a proposed pore region between transmembrane domains five and six. TRP channels are thought to have cytoplasmic N- and C-termini. The three classes of TRP channels are distinguished according to overall homology as well as a few unique characteristics. TRPV and TRPC members contain two to four N-terminal ankyrin domains thought to be involved in linking transmembrane proteins to the cytoskeleton. TRPC and TRPM members have a TRP box (with unknown function) following the sixth transmembrane domain. The involvement of TRP channels in sensory function has been evident from the beginning. It is now recognized that several other classes of ion channels are homologous to the classical TRP channels and a new nomenclature system has been proposed to reflect this relationship (Montell, 2001, *supra*). The new subtypes include TRPP for PKD2-like channels (PKD2 is mutated in polycystic kidney disease), TRPML for Mucolipidin-like channels (Mucolipidin mutations are responsible for some lysosomal storage disorders), and TRPN for NOMPC-like channels, which are distinguished by a large number of N-terminal ankyrin repeats (NOMPC is required for mechanosensory function in flies).

[0017] Pain is a major problem for the individual sufferer and for society because of the high costs involved in managing pain. Pain is often a part of numerous disorders or diseases including, for example, cancer pathology. Terminally ill subjects often suffer immensely because our ability to effectively manage pain is inadequate. Therefore, strategies to identify molecules that function in pain sensation are needed.

SUMMARY OF THE INVENTION

[0018] In one embodiment, the invention provides isolated nucleic acid sequences encoding an ANKTM1-related polypeptide, which are characterized as encoding a temperature/pain sensitive non-selective cation channel protein that is activated by temperature below 20 °C; being expressed in Calcitonin gene-related peptide- and substance P-positive neurons. The invention nucleic acid sequences comprise more than five ankyrin domains and a six transmembrane domain.

[0019] In another embodiment, the invention provides methods for identifying an agent that modulates nociceptive response by contacting an organism containing an ANKTM1-related polypeptide encoded by an invention nucleic acid sequence with an agent suspected of having nociceptive pain modulating activity under conditions that allow the agent and the polypeptide to interact. A nociceptive stimulus is then administered to the organism and any nociceptive response is measured and compared with the nociceptive response of the organism to the stimulus when not administered the agent. A change in the nociceptive response indicates the agent modulates the nociceptive response to the stimulus.

[0020] In yet another embodiment, the invention provides methods for modulating nociceptive pain in a sentient organism that contains a polypeptide sequence comprising an amino acid sequence selected from SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:4, and conservative variations thereof by contacting the organism with an effective amount of an agent that modulates operation of the polypeptide under conditions that allow the agent and the polypeptide to interact, thereby modulating nociceptive pain in the organism.

[0021] In still another embodiment, the invention provides methods for reducing nociceptive pain in an organism by contacting an organism containing an invention isolated nucleic acid sequence with an effective amount of an agent that blocks function of the polynucleotide sequence under conditions that allow the agent and the polynucleotide to interact, thereby reducing nociceptive pain in the organism.

BRIEF DESCRIPTION OF THE FIGURES

[0022] Figure 1A is a representation of a phylogenetic tree of TRP channels showing ANKTM1 grouped together with an emerging group of TRP-like channels that have multiple ankyrin domains (more than eight) and a six unit transmembrane (TM) domain. To date, ANKTM1 is the only vertebrate member of this family (mouse = mANKTM1 (SEQ ID NO:1) and human = hANKTM1 (SEQ ID NO:2). A close homologue exists in *Drosophila* that is designated dmANKTM1 (SEQ ID NO:3). Overall, four drosophila (dm) (including NOMPC) members and two *C. elegans* (ce) family members belong to this family (dmNOMPC (SEQ ID NO:4); ceNOMPC (SEQ ID NO:5); ce(NP_502249) (SEQ ID NO:6); dm(CG1-409) (SEQ ID NO:7); dm(CG17142) (SEQ ID NO:8). Similarities are calculated in Blast2.

[0023] Figure 1B is an alignment comparison of amino acid sequences from the putative transmembrane domains of proteins in the phylogenetic tree shown in Figure 1A. The alignment shows strong similarity in areas such as transmembrane domain six (TM6) among all members, and more specific pockets of similarity common to the three ANKTM1 homologues peppered throughout the region. The alignment is generated using MEGALIGN™ and BOXSHADE™ alignment programs. Identical and conserved areas are shaded. Transmembrane domains of mANKTM1 are marked as TM.

[0024] Figure 2A is a Northern blot analysis of mouse tissue showing ANKTM is not expressed in tissues tested.

[0025] Figure 2B is a Northern blot analysis of rat tissues showing expression of ANKTM1 in the dorsal root ganglia (DRG). Blots were hybridized with a ³²P-labeled fragment of ANKTM1 cDNA (top panel) and with control cDNAs (bottom panel).

[0026] Figure 3 shows the results of in-situ hybridization and immunostaining analyses, which demonstrate ANKTM1 expression in a subset of TRPV1-positive nociceptive DRG neurons.

[0027] (A-C) ANKTM1 mRNA is not present in heavily myelinated neurons marked by Neurofilament (NF 150) antibody. (D-F) ANKTM1 and calcitonin-gene-related peptide (CGRP) are coexpressed (arrows D-F). (G-L) Double-in situ hybridization shows ANKTM1 is present in a sub-population of thermosensitive neurons expressing TRPV1 (arrows G-I), but not TRPM8. Size bar is 50 μ m. (M) A schematic representation showing that ANKTM1 is expressed in a subset of TRPV1-positive nociceptive DRG neurons and in a sub-population of thermosensitive neurons expressing TRPV1, but not TRPM8.

[0028] Figure 4A is a graph showing that ANKTM1 is a calcium permeable cation channel that responds to a cold stimulus by eliciting a rise in $[Ca^{2+}]_i$ in ANKTM1-expressing CHO cells. A schematic representation of the stimulus temperature is indicated below the graph. CHO = untransfected CHO cells; RR= ruthenium red blocker. Values shown are average increase in ratio 340/380 \pm SEM of 30-40 cells from representative experiments.

[0029] Figure 4B is a graph showing average fold increase in 340 nm/380 nm ratio of ANKTM1-, TRPM8-, and TRPV1-expressing CHO cells in response to hot- (capsaicin) and cold- (menthol, icilin) inducing compounds.

[0030] Figures 5A and B show that ANKTM1 is activated at colder temperatures compared to TRPM8.

[0031] Figure 5A shows representative images taken from calcium imaging experiments comparing responses to gradual cooling of Fura-2 loaded ANKTM1- and TRPM8-expressing CHO cells. Increases in fluorescence correspond to increases in intracellular calcium levels and are indicated by a change in color where red>yellow>green>blue. Note that the majority of ANKTM1-expressing cells (left) are not active until temperatures fall below 20 $^{\circ}$ C, while many TRPM8 expressing cells (right) are already strongly activated at 20 $^{\circ}$ C.

[0032] Figure 5B is a graph showing responses of individual ANKTM1- and TRPM8-expressing CHO cells to gradual cooling from 31 °C to 9 °C. Responses shown are from representative cells.

[0033] Figures 6A-D are a series of graphs showing currents evoked by decreasing temperatures in CHO cells expressing ANKTM1.

[0034] Figure 6A provides an example of an inward current evoked by cooling of a cell voltage clamped at -60mV.

[0035] Figure 6B is a graph showing a comparison of temperature current relationship for ANKTM1 and TRPM8. The threshold for activation of ANKTM1 is lower than that of TRPM8.

[0036] Figure 6C is a graph showing current-voltage relationship for ANKTM1 (voltage ramp is from -100mV to +80mV with two second ramps). The current is outwardly rectifying and there is an increase in both inward and outward currents when the temperature is lowered (30°C-18°C).

[0037] Figure 6D is a graph showing current-voltage relationships for ANKTM1 in various external solutions. The main charge ion is (1) 140mM NaCl (2) 40mM NaCl / 100mM choline (3) 1mM CaCl (4) 30mM CaCl. E_{rev} for these examples are, +4, -6, -2 and +11mV, respectively.

[0038] Figures 7A-D are a series of graphs showing that ANKTM1 currents are desensitized to repeated cold stimuli.

[0039] Figures 7A and 7C show, respectively, inward currents recorded in response to first and second cold steps from *Xenopus* oocytes expressing ANKTM1.

[0040] Figures 7B and 7D show, respectively, inward currents recorded in response to cold steps from *Xenopus* oocytes expressing TRPM8. The oocytes were held at -70mV during the recordings. ANKTM1 current responses were markedly reduced in the second cold step.

[0041] Figure 8 shows the nucleotide sequence of the 3.378 kb mouse homologue (mANKTM) of human ANKTM (hANKTM).

DETAILED DESCRIPTION OF THE INVENTION

[0042] In humans and other vertebrates, painful stimuli are sensed by specialized neurons known as nociceptors, which fire in response to noxious temperature and mechanical or chemical stimuli, all of which have the potential to cause tissue damage. The signals are in turn processed by the central nervous system and perceived as pain, serving an indispensable protective role. Nociceptors are also involved in pathological pain states caused by inflammation, nerve damage, or cancer. An increased understanding of nociception therefore is of wide interest, and model systems for molecular genetic analysis are desirable.

[0043] The methods and compositions of the invention are based on a method, described herein, to measure nociceptive responses in vertebrates such as, for example, mammals including humans, utilizing a new member of the TRPN family that participates in thermosensation and pain.. This receptor, designated ANKTM1, is associated with nociceptive pain. Accordingly, the invention provides isolated polypeptides and polynucleotides associated with nociception as well as methods for identifying or screening agents that modulate nociception.

[0044] As used herein, "hyperalgesia" or a "hyperalgesic state" refers to a condition in which a warm-blooded animal is extremely sensitive to mechanical, chemical or thermal stimulation that, absent the condition, would be painless. Typical models for such a hyperalgesic state include the inflamed rat paw compression model and the compression of the inflamed knee joint.

[0045] Hyperalgesia is known to accompany certain physical injuries to the body, for example the injury inevitably caused by surgery. Hyperalgesia is also known to accompany certain inflammatory conditions in man such as arthritic and rheumatic disease. Hyperalgesia, thus refers to mild to moderate pain to severe pain such as the pain

associated with, but not limited to, inflammatory conditions (e.g., such as rheumatoid arthritis and osteoarthritis), postoperative pain, post-partum pain, the pain associated with dental conditions (e.g., dental caries and gingivitis), the pain associated with burns, including but not limited to sunburns, abrasions, contusions and the like, the pain associated with sports injuries and sprains, inflammatory skin conditions, including but not limited to poison ivy, and allergic rashes and dermatitis, and other such pain that increases sensitivity to mild stimuli, such as cold.

[0046] To date, all five thermo-activated channels identified belong to the TRP family, characterized by six transmembrane domains (all) and N-terminal ankyrin domains (most) (Montell, 2001, *supra*). In the invention, a combined bioinformatic and expression analysis approach has been used to identify additional TRP channels involved in sensory detection. A search in PFAM protein sequence HMM data base for predicted cDNA sequences that contain both ankyrin domains (PFAM00023) and six-transmembrane domains (PFAM00520) led to ANKTM1. Human ANKTM1 is an uncharacterized putative channel cloned from cultured fibroblasts. A previous study had shown no significant expression of ANKTM1 in various tissues examined, although EST sequences suggest that ANKTM1 is upregulated in many human tumor cells (Jaquemar et al., *J Biol Chem* 274:7325-7333, 1999).

[0047] To characterize human ANKTM1, full-length mouse ANKTM1 was amplified from mouse dorsal root ganglia (DRG) and trigeminal ganglia that contain somatic sensory neurons using RT-PCR. Theoretical translation of the mouse nucleotide sequence predicts a protein of 1125 amino acid residues (SEQ ID NO:1), very similar to human ANKTM1 (1119 amino acids) (SEQ ID NO:2) (Figure 1A). Mouse ANKTM1 has 14 predicted N-terminal ankyrin domains followed by a six transmembrane (6TM) domain.

[0048] A phylogenetic tree analysis of all TRP-like channels grouped ANKTM1 with *Drosophila* NOMPC, which has 29 N-terminal ankyrin domains and is required for mechanosensation (Walker et al., *Science* 287:2229-2234, 2000). Further searches for proteins that contain both a 6TM domain and more than five ankyrin domains (to exclude

TRPV and TRPC members, which have two to four ankyrin domains) demonstrated that ANKTM1 is the only mammalian member of this group. *Drosophila* has four such proteins dmANKTM1 (SEQ ID NO:3, dmNOMPC (SEQ ID NO:4), dmCG10409 (SEQ ID NO:7) and dmCG17142 (SEQ ID NO:8). In *C. elegans* two such proteins have been found, ceNOMPC (SEQ ID NO:5) and ceNP-502249 (SEQ ID NO:6) (Figures 1A and B). These putative channels form a branch of TRP proteins named TRPN after the founding member, NOMPC. Some of the TRPN family members do not have high similarity at the primary amino acid level: mANKTM1 (SEQ ID NO:1) shares only 25% amino acid identity to dmNOMPC (SEQ ID NO:4). By comparison, TRPV1 and TRPV3 are 43% similar. The clustering of mANKTM1 and NOMPC in the phylogenetic tree reflects their shared numerous N-terminal ankyrin domains. Indeed, if 6TM domains alone are compared, ANKTM1 is more similar to TRPV3 than to NOMPC. Therefore, the phylogenetic tree organization should be carefully interpreted.

[0049] However, at the amino-acid level, the two cold-receptors surprisingly have no significant amino acid sequence identity. This finding is in contrast to the four heat-activated TRPV channels, which have at least 40% amino acid sequence identity. The transmembrane domain of ANKTM1 is more similar to TRPV members than to any other mammalian proteins. This amino acid similarity is likely significant at the structural level since ruthenium red, a potent blocker of all TRPV channels, also blocks ANKTM1. The 16 N-terminal ankyrin domains show further correlation with TRPV channels (two to four ankyrin domains). However, in phylogenetic tree prediction programs, ANKTM1 is grouped with the emerging TRPN family of NOMPC-like channels, probably due to the large number of N-terminal ankyrin domains reported in this family. Within this group of putative channels, the uncharacterized protein CG5751 is shown to be the *Drosophila* orthologue of ANKTM1, since the two proteins are more similar to each other than to any other protein.

[0050] Most TRP channels are non-selective cation channels and readily let calcium into cells when activated, permitting both electrophysiological studies and intracellular calcium imaging. Therefore, in the studies described herein a series of intracellular

calcium imaging and electrophysiological studies were applied to ANKTM1. Fluorescence microscopy was used to monitor increases in intracellular calcium concentration in response to various sensory stimuli by loading ANKTM1-expressing CHO cells with the calcium indicator fluorescent dye, Fura-2. Hypo-osmotic solutions are known to elicit calcium influx in TRPV4-expressing cells. However, when ANKTM1 was subjected to hypo-osmotic solution, activation did not occur. Heat stimuli of up to 42 °C and 52 °C are strong activators of TRPV1 and TRPV2 respectively (Caterina et al., 1999, supra; Caterina et al., 1997, supra). However, ANKTM1-expressing CHO cells, when stimulated with heat in this range, were not activated. Menthol or cool stimuli in the range of 23-28 °C, the threshold of activation of TRPM8 (McKemy et al., 2002, supra; Peier et al., 2002b, supra), also failed to activate ANKTM1.

[0051] However, it was discovered that lowering temperature to 10 °C caused a large influx of calcium in ANKTM1-expressing cells, but not in Tet-treated CHO cell controls (Figure 4A). This response to cold stimulus by ANKTM1 was abolished upon removal of extracellular calcium, showing that ANKTM1 is acting as a calcium permeable channel rather than releasing calcium from intracellular stores (Figure 4A). Similar responses to cold were obtained from CHO cells transiently transfected with ANKTM1 and from Tet-inducible human embryonic kidney (HEK 293) cells stably expressing ANKTM1.).

[0052] Ruthenium red, a blocker of thermosensitive TRPV channels, also blocked cold responses by ANKTM1. Pre-incubation of ANKTM1-expressing cells in 5µM ruthenium red followed by application of cold completely blocked responses, which were not entirely recovered after a ten minute washout (Figure 4). Similar experiments performed with 1µM ruthenium red caused complete block of the cold response in 90% of ANKTM1-containing cells from multiple experiments.

[0053] Icilin, an ultra- cooling agent and potent activator of TRPM8, also activated ANKTM1-expressing cells (Figure 4B) (McKemy et al., 2002, supra), but Icilin did not activate TRPV1-expressing cells. In additional tests, capsaicin, a potent activator of TRPV1, did not activate TRPM8- or ANKTM1-expressing cells (Figure 4B). Higher concentrations of Icilin were required for ANKTM1 activation compared to TRPM8, and

the response was relatively delayed in ANKTM1 cells. There was a 15-60 second delay for ANKTM1 as compared with a 15 seconds delay for TRPM8. Therefore, it is not clear if Icilin directly binds to and activates ANKTM1.

[0054] These experiments show that both TRPM8 and ANKTM1 respond to cold. However, to confirm that threshold for activation of ANKTM1 is lower than that of TRPM8, as suggested in the above-described experimental results, further tests were conducted. The activation temperature of TRPM8 is reported at approximately 23 °C (Peier et al., 2002a, supra). Therefore, to further compare the activation temperatures of ANKTM1 and TRPM8, calcium imaging experiments were conducted wherein incubation temperature of both types of cells was elevated to 33 °C for several minutes before the bath temperature was lowered gradually to 10 °C. The majority of TRPM8-expressing cells exhibited increased intracellular calcium concentration when the temperature was lowered to 20 °C (Figure 5A). However, the majority of ANKTM1-expressing cells were not activated until the temperature was cooled to 10 °C (Figure 5A). Quantitative analysis showed that TRPM8-expressing cells exhibited an activation threshold (defined as 20% increase from baseline fluorescence) at temperatures ranging from 19-24 °C with a mean activation temperature of 22.5 ± 1 °C (mean \pm SD, n=60). By comparison, ANKTM1-expressing cells exhibited a broader range of activation temperatures (12 °C-24 °C) with an average activation temperature of 17.5 ± 3.5 °C (mean \pm SD, n=100). Figure 5B illustrates traces of typical responses of individual ANKTM1 and TRPM8-expressing cells as temperature is gradually decreased.

[0055] ANKTM1- and TRPM8-expressing CHO cells were also assayed electrophysiologically using the whole cell voltage clamp technique. The cells were clamped at -60mV and the temperature of the perfused bath solution was decreased from 32 °C to 10 °C. In ANKTM1-expressing cells small and slowly developing inward currents were observed followed by rapid and larger phase currents with an average peak amplitude of 0.55 ± 0.07 nA (n= 35) (Figure 6A and 6B). The activation temperature for the large evoked currents varied from cell to cell, ranging from 8-28 °C, yielding results similar to those obtained by calcium imaging (n=35). A comparison of representative

currents from ANKTM1- and TRPM8-expressing CHO cells shown in Figure 6B demonstrates a lower threshold of activation for ANKTM1 than for TRPM8.

[0056] Currents evoked by decreasing the temperature in ANKTM1-expressing cells show outward rectification, but with substantial current in the inward direction (Figures 6C and 6D). A reversal potential of $+7.7 \pm 1.2$ mV was observed in an external solution containing 140 mM NaCl ($n=6$). Reducing the NaCl in the external solution to 40 mM (by equimolar replacement with 100 mM choline chloride) caused a negative shift in the reversal potential. This result is consistent with ANKTM1 being a cation channel. Differences in reversal potentials were used to determine the ionic selectivity of ANKTM1. The shift in reversal potential of -18mV to -10.35 ± 1.6 mV ($n=7$) seen upon replacing 100 mM NaCl with 100 mM choline Cl gives a relative permeability ratio of $P_{\text{choline}}/P_{\text{Na}} = 0.28$. The reversal potentials of the cold-activated currents were similar in simplified external solutions containing 100 mM Choline and 40 mM NaCl, KCl, or CsCl. The measured reversal potentials yield relative permeability ratios of $P_{\text{K}}/P_{\text{Na}} = 1.19$ and $P_{\text{Cs}}/P_{\text{Na}} = 1.43$ (NaCl, $E_{\text{rev}} = -10.35 \pm 1.6$ mV, $n=7$; KCl, $E_{\text{rev}} = -7.65 \pm 1.5$ mV, $n=8$; CsCl, $E_{\text{rev}} = -4.75 \pm 0.75$ mV, $n=7$). The relative permeability of Ca^{2+} and Mg^{2+} were estimated from the shift in reversal potentials when their concentrations were raised from 1 mM to 30 mM in a 40 mM NaCl /100 mM choline Cl solution containing the divalent cation under investigation. In these tests, the reversal potential shifted from $+0.54 \pm 3.3$ (1mM CaCl_2 , $n=6$) to $+14.16 \pm 3.9$ mV (30mM CaCl_2 , $n=6$) for Ca^{2+} and from -14.36 ± 1.1 (1mM MgCl_2 , $n=6$) to $+7.01 \pm 2.9$ mV (30mM MgCl_2 , $n=6$) for Mg^{2+} , corresponding to $P_{\text{Ca}}/P_{\text{Na}} = 0.84$ and $P_{\text{Mg}}/P_{\text{Na}} = 1.23$ (Figure 6D). These results indicate that ANKTM1 is a non-selective cation permeable channel, similar to many previously described TRP channels.

[0057] To investigate the properties of ANKTM1 in another heterologous system, *Xenopus* oocytes were injected with ANKTM1 cRNA. Large currents were observed in response to cold temperatures, similar to the activity of ANKTM1-expressing CHO cells (Figures 7A and C). In both *Xenopus* and CHO systems, a strong desensitization of ANKTM1 to cold stimuli was observed. Cold activation of ANKTM1 showed a marked

desensitization during a first cold pulse, and desensitization to repeated cold pulses (Figures 7B and D). On average, the second cold pulse resulted in a current that was 26% of the first pulse in ANKTM1-injected oocytes (SD=6.5, n=5), compared to 78% for TRPM8 (SD=5.9, n=4).

[0058] The above-described characterization studies show that ANKTM1 belongs to the superfamily of TRP channels as does the menthol- and cold-activated receptor, TRPM8, despite the lack of amino acid sequence similarity between the two. Functional studies of ANKTM1 agree with the phylogenetic characterization of this channel as TRP-like. There is now strong evidence that a group of TRP channels play a role in temperature detection in the mammalian peripheral nervous system. ANKTM1 belongs to the third TRP subfamily shown to play a role in temperature detection. Like other thermosensitive TRPs, ANKTM1 is a non-selective cation channel. However, ANKTM1 displays several unique characteristics compared to previously characterized temperature-activated TRP channels. The variability in activation threshold temperature of ANKTM1 from cell to cell is broader when compared to other TRPs. Furthermore, the current through ANKTM1 rapidly desensitizes to cold, a property not seen to such an extent in other temperature-activated TRPs. Finally, long-term overexpression of ANKTM1 is detrimental to cells, making it necessary to for cell lines to conditionally express ANKTM1. The mechanisms underlying the threshold variability, strong desensitization, and cell toxicity are not yet clear.

[0059] ANKTM1 is activated at lower temperatures than TRPM8, starting at near 17 °C, which approximates the threshold of noxious cold for humans (~ 15 °C). A role for ANKTM1 in noxious cold detection is also suggested by its expression pattern. Mouse ANKTM1 is specifically expressed in somatic sensory neurons. Within this population, ANKTM1 is not expressed in neurons that express TRPM8. Instead, the vast majority of ANKTM1-positive cells also express TRPV1 and CGRP, markers for pain-sensing neurons. Recent reports describe the presence of two separate populations of cold-sensitive DRG neurons: one population that expresses TRPM8 and is menthol-sensitive, and a distinct population that is menthol-insensitive and is activated at even

colder temperatures. It is likely that ANKTM1 marks this second population of cold sensitive neurons.

[0060] The major overlap of ANKTM1 and TRPV1 tissue expression raises the question of how temperature/pain information is decoded in the brain and spinal cord. Electrophysiological characterization of DRG neurons has led to the labeled line hypothesis of sensory perception (Scott, 1992). According to this hypothesis, the nervous system interprets the environment by monitoring the electrical activity of distinct groups of sensory neurons that are specialized to detect a unique sensory modality. However, this hypothesis does not incorporate the observation of single neurons known to transmit more than one sensory modality, for example in this case cold and pain. Indeed, there are so-called polymodal nociceptors that respond to a variety of noxious stimuli (Julius and Basbaum, *Nature* 413:203-210,2001). Here molecular evidence is shown that a cold-activated channel (ANKTM1) is expressed in a subset of heat-sensitive (TRPV1-expressing) neurons.

[0061] The identification of ANKTM1 as a channel activated by noxious cold increases the range of temperatures "sensed" by TRP channels, but does not explain how the quality of the sensation changes from pleasantly cool to aching or burning, i.e., pain, depending on whether ANKTM1, TRPM8, or both of these cold channels are active.

[0062] The term "isolated" means altered "by the hand of man" from its natural state; i.e., if it occurs in nature, it has been changed or removed from its original environment, or both. For example, a naturally occurring polynucleotide or a polypeptide naturally present in a living animal in its natural state is not "isolated", but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein. As part of or following isolation, a polynucleotide can be joined to other polynucleotides, such as for example DNAs, for mutagenesis studies, to form fusion proteins, and for propagation or expression of the polynucleotide in a host. The isolated polynucleotides, alone or joined to other polynucleotides, such as vectors, can be introduced into host cells, in culture or in whole organisms. Such polynucleotides, when introduced into host cells in culture or in whole organisms, still would be isolated,

as the term is used herein, because they would not be in their naturally occurring form or environment. Similarly, the polynucleotides and polypeptides may occur in a composition, such as a media formulation (solutions for introduction of polynucleotides or polypeptides, for example, into cells or compositions or solutions for chemical or enzymatic reactions).

[0063] "Polynucleotide" or "nucleic acid sequence" refers to a polymeric form of nucleotides. In some instances a polynucleotide refers to a sequence that is not immediately contiguous with either of the coding sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally occurring genome of the organism from which it is derived. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (*e.g.*, a cDNA) independent of other sequences. The nucleotides of the invention can be ribonucleotides, deoxyribonucleotides, or modified forms of either nucleotide. In addition, the polynucleotide sequence involved in producing a polypeptide chain can include regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons) depending upon the source of the polynucleotide sequence.

[0064] The term "polynucleotide(s)" generally refers to any polyribonucleotide or polydeoxyribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. Thus, for instance, polynucleotides as used herein refers to, among others, single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions.

[0065] . In addition, polynucleotide as used herein refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The strands in such regions may be from the same molecule or from different molecules. The regions may include all of one

or more of the molecules, but more typically involve only a region of some of the molecules. One of the molecules of a triple-helical region often is an oligonucleotide.

[0066] In addition, the polynucleotides or nucleic acid sequences may contain one or more modified bases. Thus, DNAs or RNAs with backbones modified for stability or for other reasons are "polynucleotides" as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritylated bases, to name just two examples, are polynucleotides as the term is used herein.

[0067] Nucleic acid sequences can be created which encode a fusion protein and can be operatively linked to expression control sequences. "Operatively linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. For example, a coding sequence is "operably linked" to another coding sequence when RNA polymerase will transcribe the two coding sequences into a single mRNA, which is then translated into a single polypeptide having amino acids derived from both coding sequences. The coding sequences need not be contiguous to one another so long as the expressed sequences ultimately process to produce the desired protein. An expression control sequence operatively linked to a coding sequence is ligated such that expression of the coding sequence is achieved under conditions compatible with the expression control sequences. As used herein, the term "expression control sequences" refers to nucleic acid sequences that regulate the expression of a nucleic acid sequence to which it is operatively linked. Expression control sequences are operatively linked to a nucleic acid sequence when the expression control sequences control and regulate the transcription and, as appropriate, translation of the nucleic acid sequence. Thus, expression control sequences can include appropriate promoters, enhancers, transcription terminators, a start codon (*i.e.*, ATG) in front of a protein-encoding gene, splicing signals for introns, maintenance of the correct reading frame of that gene to permit proper translation of the mRNA, and stop codons. The term "control sequences" is intended to include, at a minimum, components whose presence can influence expression, and can also include additional components whose presence is

advantageous, for example, leader sequences and fusion partner sequences. Expression control sequences can include a promoter.

[0068] By "promoter" is meant minimal sequence sufficient to direct transcription. Also included in the invention are those promoter elements which are sufficient to render promoter-dependent gene expression controllable for cell-type specific, tissue-specific, or inducible by external signals or agents; such elements may be located in the 5' or 3' regions of the of the polynucleotide sequence. Both constitutive and inducible promoters, are included in the invention (see *e.g.*, Bitter *et al.*, Methods in Enzymology 153:516-544, 1987). For example, when cloning in bacterial systems, inducible promoters such as *pL* of bacteriophage, *plac*, *ptrp*, *ptac* (*ptrp-lac* hybrid promoter) and the like may be used. When cloning in mammalian cell systems, promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the retrovirus long terminal repeat; the adenovirus late promoter; the vaccinia virus 7.5K promoter) may be used. Promoters produced by recombinant DNA or synthetic techniques may also be used to provide for transcription of the nucleic acid sequences of the invention.

[0069] A nucleic acid sequence of the invention including, for example, a polynucleotide encoding a fusion protein, may be inserted into a recombinant expression vector. A recombinant expression vector generally refers to a plasmid, virus or other vehicle known in the art that has been manipulated by insertion or incorporation of a nucleic acid sequences. For example, a recombinant expression vector of the invention includes a polynucleotide sequence encoding an ANKTM1-related polypeptide involved in nociception or a fragment thereof. The expression vector typically contains an origin of replication, a promoter, as well as specific genes that allow phenotypic selection of the transformed cells. Vectors suitable for use in the invention include, but are not limited to the T7-based expression vector for expression in bacteria (Rosenberg, *et al.*, Gene 56:125, 1987), the pMSXND expression vector for expression in mammalian cells (Lee and Nathans, J. Biol. Chem. 263:3521, 1988), baculovirus-derived vectors for expression in insect cells, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV. The nucleic

acid sequences of the invention can also include a localization sequence to direct the indicator to particular cellular sites by fusion to appropriate organellar targeting signals or localized host proteins. For example, a polynucleotide encoding a localization sequence, or signal sequence, can be used as a repressor and thus can be ligated or fused at the 5' terminus of a polynucleotide encoding a polypeptide of the invention such that the localization or signal peptide is located at the amino terminal end of a resulting polynucleotide/polypeptide. The construction of expression vectors and the expression of genes in transfected cells involves the use of molecular cloning techniques also well known in the art. (See, for example, Sambrook *et al.*, Molecular Cloning --A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989, and Current Protocols in Molecular Biology, M. Ausubel *et al.*, eds., (Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., most recent Supplement)). These methods include *in vitro* recombinant DNA techniques, synthetic techniques and *in vivo* recombination/genetic recombination. (See also, Maniatis, *et al.*, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y., 1989).

[0070] In yeast, a number of vectors containing constitutive or inducible promoters may be used. For a review, see Current Protocols in Molecular Biology, Vol. 2, Ed. Ausubel, *et al.*, Greene Publish. Assoc. & Wiley Interscience, Ch. 13, 1988; Grant, *et al.*, "Expression and Secretion Vectors for Yeast," in Methods in Enzymology, Eds. Wu & Grossman, 1987, Acad. Press, N.Y., Vol. 153, pp. 516-544, 1987; Glover, DNA Cloning, Vol. II, IRL Press, Wash., D.C., Ch. 3, 1986; and Bitter, "Heterologous Gene Expression in Yeast," Methods in Enzymology, Eds. Berger & Kimmel, Acad. Press, N.Y., Vol. 152, pp. 673-684, 1987; and The Molecular Biology of the Yeast *Saccharomyces*, Eds. Strathern *et al.*, Cold Spring Harbor Press, Vols. I and II, 1982. A constitutive yeast promoter such as *ADH* or *LEU2* or an inducible promoter such as *GAL* may be used ("Cloning in Yeast," Ch. 3, R. Rothstein In: DNA Cloning Vol.11, A Practical Approach, Ed. DM Glover, IRL Press, Wash., D.C., 1986). Alternatively, vectors may be used which promote integration of foreign DNA sequences into the yeast chromosome.

[0071] An alternative expression system that could be used to express an invention ANKTM1-related polypeptide is an insect system. In one such system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign or mutated polynucleotide sequences. The virus grows in *Spodoptera frugiperda* cells. The sequence encoding an invention protein may be cloned into non-essential regions (for example, the polyhedrin gene) of the virus and placed under control of an *AcNPV* promoter (for example the polyhedrin promoter). Successful insertion of the sequences coding for a protein of the invention will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (*i.e.*, virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect *S. frugiperda* cells in which the inserted gene is expressed, see Smith, *et al.*, J. Viol. 46:584, 1983; Smith, U.S. Patent No. 4,215,051.

[0072] The vectors of the invention can be used to transform a host cell. By transform or transformation is meant a permanent or transient genetic change induced in a cell following incorporation of new DNA (*i.e.*, DNA exogenous to the cell). Where the cell is a mammalian cell, a permanent genetic change is generally achieved by introduction of the DNA into the genome of the cell.

[0073] A transformed cell or host cell generally refers to a cell (*e.g.*, prokaryotic or eukaryotic) into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a DNA molecule encoding an invention ANKTM1-related polypeptide or a fragment thereof.

[0074] Transformation of a host cell with recombinant DNA may be carried out by conventional techniques as are well known to those skilled in the art. Where the host is prokaryotic, such as *E. coli*, competent cells which are capable of DNA uptake can be prepared from cells harvested after exponential growth phase and subsequently treated by the CaCl_2 method by procedures well known in the art. Alternatively, MgCl_2 or RbCl can be used. Transformation can also be performed after forming a protoplast of the host cell or by electroporation.

[0075] When the host is a eukaryote, methods for transfection or transformation with DNA include calcium phosphate co-precipitates, conventional mechanical procedures such as microinjection, electroporation, insertion of a plasmid encased in liposomes, or virus vectors, as well as others known in the art, may be used. Eukaryotic cells can also be cotransfected with DNA sequences encoding an invention ANKTM1-related polypeptide and a second foreign DNA molecule encoding a selectable marker, such as the herpes simplex thymidine kinase gene. Another method is to use a eukaryotic viral vector, such as simian virus 40 (SV40) or bovine papilloma virus, to transiently infect or transform eukaryotic cells and express the protein. (Eukaryotic Viral Vectors, Cold Spring Harbor Laboratory, Gluzman ed., 1982). Typically, a eukaryotic host will be utilized as the host cell. The eukaryotic cell may be a yeast cell (*e.g.*, *Saccharomyces cerevisiae*), an insect cell (*e.g.*, *Drosophila sp.*) or may be a mammalian cell, including a human cell.

[0076] Eukaryotic systems, and mammalian expression systems, allow for post-translational modifications of expressed mammalian proteins to occur. Eukaryotic cells which possess the cellular machinery for processing of the primary transcript, glycosylation, phosphorylation, and, advantageously secretion of the gene product should be used. Such host cell lines may include, but are not limited to, CHO, VERO, BHK, HeLa, COS, MDCK, Jurkat, HEK-293, and WI38.

[0077] Mammalian cell systems that utilize recombinant viruses or viral elements to direct expression may be engineered. For example, when using adenovirus expression vectors, a polynucleotide encoding an invention ANKTM1-related polypeptide may be ligated to an adenovirus transcription/ translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric sequence may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing an invention ANKTM1-related polypeptide or a fragment thereof in infected hosts (*e.g.*, see Logan & Shenk, Proc. Natl. Acad. Sci. USA, 81:3655-3659, 1984). Alternatively, the vaccinia virus 7.5K promoter may be used.

(e.g., see, Mackett, *et al.*, Proc. Natl. Acad. Sci. USA, 79:7415-7419, 1982; Mackett, *et al.*, J. Virol. 49:857-864, 1984; Panicali, *et al.*, Proc. Natl. Acad. Sci. USA 79:4927-4931, 1982). Of particular interest are vectors based on bovine papilloma virus, which have the ability to replicate as extrachromosomal elements (Sarver, *et al.*, Mol. Cell. Biol. 1:486, 1981). Shortly after entry of this DNA into mouse cells, the plasmid replicates to about 100 to 200 copies per cell. Transcription of the inserted cDNA does not require integration of the plasmid into the host's chromosome, thereby yielding a high level of expression. These vectors can be used for stable expression by including a selectable marker in the plasmid, such as the *neo* gene. Alternatively, the retroviral genome can be modified for use as a vector capable of introducing and directing the expression of an invention nociception-related gene in host cells (Cone & Mulligan, Proc. Natl. Acad. Sci. USA, 81:6349-6353, 1984). High-level expression may also be achieved using inducible promoters, including, but not limited to, the metallothionein IIA promoter and heat shock promoters.

[0078] For long-term, high-yield production of recombinant proteins, stable expression is preferred. Rather than using expression vectors that contain viral origins of replication, host cells can be transformed with the cDNA encoding an invention ANKTM1-related polypeptide controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. The selectable marker in the recombinant vector confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci, which in turn can be cloned and expanded into cell lines. For example, following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. A number of selection systems may be used, including, but not limited to, the herpes simplex virus thymidine kinase (Wigler, *et al.*, Cell, 11:223, 1977), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, Proc. Natl. Acad. Sci. USA, 48:2026, 1962), and adenine phosphoribosyltransferase (Lowy, *et al.*, Cell, 22:817, 1980) genes can be employed in *tk*-, *hgp*rt- or *ap*rt- cells respectively. Also, anti-metabolite resistance can be used as the basis of selection for dhfr, which confers resistance to

methotrexate (Wigler, *et al.*, Proc. Natl. Acad. Sci. USA, 77:3567, 1980; O'Hare, *et al.*, Proc. Natl. Acad. Sci. USA, 8:1527, 1981); *gpt*, which confers resistance to mycophenolic acid (Mulligan & Berg, Proc. Natl. Acad. Sci. USA, 78:2072, 1981; *neo*, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, *et al.*, J. Mol. Biol. 150:1, 1981); and *hygro*, which confers resistance to hygromycin (Santerre, *et al.*, Gene 30:147, 1984) genes. Recently, additional selectable genes have been described, namely *trpB*, which allows cells to utilize indole in place of tryptophan; *hisD*, which allows cells to utilize histinol in place of histidine (Hartman & Mulligan, Proc. Natl. Acad. Sci. USA 85:8047, 1988); and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue L., In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory, ed., 1987).

[0079] The term "primer" as used herein refers to an oligonucleotide, whether natural or synthetic, which is capable of acting as a point of initiation of synthesis when placed under conditions in which primer extension is initiated or possible. Synthesis of a primer extension product that is complementary to a nucleic acid strand is initiated in the presence of nucleoside triphosphates and a polymerase in an appropriate buffer at a suitable temperature. For instance, if a nucleic acid sequence is inferred from a protein sequence, a primer generated to synthesize nucleic acid sequence encoding the protein sequence is actually a collection of primer oligonucleotides containing sequences representing all possible codon variations based on the degeneracy of the genetic code. One or more of the primers in this collection will be homologous with the end of the target sequence. Likewise, if a "conserved" region shows significant levels of polymorphism in a population, mixtures of primers can be prepared that will amplify adjacent sequences.

[0080] A polypeptide or protein refers to a polymer in which the monomers are amino acid residues that are joined together through amide bonds. When the amino acids are alpha-amino acids, either the L-optical isomer or the D-optical isomer can be used, the L-isomers being typical. An invention ANKTM1-related polypeptide is intended to

encompass any amino acid sequence and include modified sequences such as glycoproteins, which provides a polypeptide having nociception modulating activity. Accordingly, the polypeptides of the invention are intended to cover naturally occurring proteins, as well as those that are recombinantly or synthetically synthesized. In addition, an invention ANKTM1-related polypeptide can occur in at least two different conformations wherein both conformations have the same or substantially the same amino acid sequence but have different three-dimensional structures so long as they have a biological activity related to nociception. Polypeptide or protein fragments are also encompassed by the invention. Fragments can have the same or substantially the same amino acid sequence as the naturally occurring protein. A polypeptide or peptide having substantially the same sequence means that an amino acid sequence is largely, but not entirely, the same, but retains a functional activity of the sequence to which it is related. In general polypeptides of the invention include peptides, or full-length protein, that contains substitutions, deletions, or insertions into the protein backbone, that would still have an approximately 70%-90% homology to the original protein over the corresponding portion. A yet greater degree of departure from homology is allowed if like-amino acids, *i.e.* conservative amino acid substitutions, do not count as a change in the sequence

[0081] A polypeptide may be substantially related but for a conservative variation, such polypeptides being encompassed by the invention. A conservative variation denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative variations include the substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acids, or glutamine for asparagine, and the like. Other illustrative examples of conservative substitutions include the changes of: alanine to serine; arginine to lysine; asparagine to glutamine or histidine; aspartate to glutamate; cysteine to serine; glutamine to asparagine; glutamate to aspartate; glycine to proline; histidine to asparagine or glutamine; isoleucine to leucine or valine; leucine to valine or isoleucine; lysine to arginine, glutamine, or glutamate; methionine to leucine or isoleucine; phenylalanine to tyrosine, leucine or methionine; serine to threonine; threonine to serine; tryptophan to

tyrosine; tyrosine to tryptophan or phenylalanine; valine to isoleucine to leucine. The term "conservative variation" also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid provided that antibodies raised to the substituted polypeptide also immunoreact with the unsubstituted polypeptide.

[0082] Modifications and substitutions are not limited to replacement of amino acids. For a variety of purposes, such as increased stability, solubility, or configuration concerns, one skilled in the art will recognize the need to introduce, (by deletion, replacement, or addition) other modifications. Examples of such other modifications include incorporation of rare amino acids, dextra-amino acids, glycosylation sites, cytosine for specific disulfide bridge formation. The modified peptides can be chemically synthesized, or the isolated gene can be site-directed mutagenized, or a synthetic gene can be synthesized and expressed in bacteria, yeast, baculovirus, tissue culture and so on.

[0083] In one embodiment, the invention provides an isolated polynucleotide sequence encoding an invention ANKTM1-related polypeptide. An invention ANKTM1-related polypeptide can be characterized by its ability to modulate nociceptive responses. Polynucleotide sequences of the invention include DNA, cDNA and RNA sequences that encode a nociceptive-related polypeptide of the invention as well as complementary sequences thereof. It is understood that all polynucleotides encoding all or a portion of an invention ANKTM1-related polypeptide are also included herein, so long as they encode a polypeptide with nociceptive activity (e.g., modulation of nociceptive responses). Such polynucleotides include naturally occurring, synthetic, and intentionally manipulated polynucleotides. For example, a nociceptive polynucleotide of the invention may be subjected to site-directed mutagenesis. The polynucleotides of the invention include sequences that are degenerate as a result of the genetic code. There are 20 natural amino acids, most of which are specified by more than one codon. Therefore, all degenerate nucleotide sequences are included in the invention so long as the amino acid sequence of an invention ANKTM1-related polypeptide encoded by the nucleotide sequence is functionally unchanged. In addition, polypeptide fragments of an invention ANKTM1-related polypeptide, and their corresponding polynucleotide sequences are encompassed

by the current invention, so long as the polypeptides retain some biological activity related to nociception. A biological activity related to nociception includes for example, antigenicity or the ability to modulate nociceptive responses. Assays described in the examples below are capable of identifying such fragments or modified polypeptides having a biological activity related to nociception. For example, a polypeptide that modulates a nociceptive response (*e.g.*, intracellular calcium imaging tests) is encompassed by the invention whether it is expressed *in vivo* by the organism or administered to the organism.

[0084] The polynucleotides and polypeptides of this invention were originally recovered from human and mouse tissue. Thus, the present invention provides means for isolating the nucleic acid molecules from other organisms, encoding the invention ANKTM1-related polypeptides. For example, one may probe a gene library with a natural or artificially designed probe using art recognized procedures (see, for example: Current Protocols in Molecular Biology, Ausubel F.M. *et al.* (Eds.) Green Publishing Company Assoc. and John Wiley Interscience, New York, 1989, 1992). It is appreciated by those skilled in the art that probes can be designed based on the degeneracy of the genetic code to a sequences corresponding to a polypeptide or polynucleotide of the invention.

[0085] In addition, sequencing algorithms can be used to measure homology or identity between known and unknown sequences. Such methods and algorithms are useful in identifying corresponding sequences present in other organisms. Homology or identity is often measured using sequence analysis software (*e.g.*, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). Such software matches similar sequences by assigning degrees of homology to various deletions, substitutions and other modifications. The terms "homology" and "identity" in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same when compared and aligned for maximum

correspondence over a comparison window or designated region as measured using any number of sequence comparison algorithms or by manual alignment and visual inspection.

[0086] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

[0087] Methods for alignment of sequence for comparison are well known in the art. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method for Person & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection.

[0088] One example of a useful algorithm is BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.*, *Nuc. Acids Res.* 25:3389-3402 (1997) and Altschul *et al.*, *J. Mol. Biol.* 215:403-410 (1990), respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, *e.g.*, Karlin & Altschul, *Proc. Natl. Acad. Sci. USA* 90:5873 (1993)). Other algorithms for determining homology or identity are well known in the art. Such alignment programs can also be used to screen genome databases to identify polynucleotide sequences having substantially identical sequences.

[0089] A "substantially pure polypeptide" is typically pure when it is at least 60%, by weight, free from the proteins and naturally occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, an invention ANKTM1-related polypeptide. A substantially pure ANKTM1-related polypeptide may be obtained, for example, by extraction from a natural source (*e.g.*, a mammalian cell); by expression of a recombinant nucleic acid encoding an ANKTM1-related polypeptide; or by chemically synthesizing the protein. Purity can be measured by any appropriate method, *e.g.*, by column chromatography, polyacrylamide gel electrophoresis, or by HPLC analysis.

[0090] In addition to polypeptides of the invention, specifically disclosed herein is a DNA sequence for an invention ANKTM1-related polypeptide. DNA sequences of the invention can be obtained by several methods. For example, the DNA can be isolated using hybridization or computer-based techniques that are well known in the art. These include, but are not limited to: 1) hybridization of genomic libraries with probes to detect homologous nucleotide sequences; 2) antibody screening of expression libraries to detect cloned DNA fragments with shared structural features; 3) polymerase chain reaction (PCR) on genomic DNA using primers capable of annealing to the DNA sequence of interest; and 4) computer searches of sequence databases for similar sequences as described above.

[0091] The polynucleotide encoding an invention ANKTM1-related polypeptide includes complementary polynucleotide sequences, as well as splice variants thereof. When the sequence is RNA, the deoxyribonucleotides A, G, C, and T are replaced by ribonucleotides A, G, C, and U, respectively. Also included in the invention are fragments (portions) of the above-described nucleic acid sequences that are at least 15 bases in length, which is sufficient to permit the fragment to selectively hybridize to DNA that encodes a polypeptide sequence of the invention. "Selective hybridization" as used herein refers to hybridization under moderately stringent or highly stringent physiological conditions (See, for example, the techniques described in Maniatis *et al.*, 1989 Molecular

Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y., incorporated herein by reference), which distinguishes related from unrelated nucleotide sequences.

[0092] In nucleic acid hybridization reactions, the conditions used to achieve a particular level of stringency will vary, depending on the nature of the nucleic acids being hybridized. For example, the length, degree of complementarity, nucleotide sequence composition (*e.g.*, GC v. AT content), and nucleic acid type (*e.g.*, RNA v. DNA) of the hybridizing regions of the nucleic acids can be considered in selecting hybridization conditions. An additional consideration is whether one of the nucleic acids is immobilized, for example, on a filter.

[0093] An example of progressively higher stringency conditions is as follows: 2 x SSC/0.1% SDS at about room temperature (hybridization conditions); 0.2 x SSC/0.1% SDS at about room temperature (low stringency conditions); 0.2 x SSC/0.1% SDS at about 42 °C (moderate stringency conditions); and 0.1 x SSC at about 68 °C (high stringency conditions). Washing can be carried out using only one of these conditions, *e.g.*, high stringency conditions, or each of the conditions can be used, *e.g.*, for 10-15 minutes each, in the order listed above, repeating any or all of the steps listed. However, as mentioned above, optimal conditions will vary, depending on the particular hybridization reaction involved, and can be determined empirically.

[0094] Oligonucleotides encompassed by the present invention are also useful as primers for nucleic acid amplification reactions. In general, the primers used according to the method for the invention embrace oligonucleotides of sufficient length and appropriate sequence to provide specific initiation of polymerization of a significant number of nucleic acid molecules containing the target nucleic acid under the conditions of stringency for the reaction utilizing the primers. In this manner, it is possible to selectively amplify the specific target nucleic acid sequence containing the nucleic acid of interest.

[0095] Amplified products may be detected by Southern blot analysis, without using radioactive probes. In such a process, for example, a small sample of DNA containing a

very low level of a nucleotide sequence is amplified and analyzed via a Southern blotting technique known to those of skill in the art. The use of non-radioactive probes or labels is facilitated by the high level of the amplified signal.

[0096] Screening procedures that rely on nucleic acid hybridization make it possible to isolate any gene sequence from any organism, provided the appropriate probe is available. For example, it is envisioned that such probes can be used to identify other homologs of the ANKTM1-related polynucleotide family of factors in mammals or, alternatively, in other organisms such as invertebrates. In accomplishing this, alignment algorithms (as described above) can be used to screen genome databases. Alternatively, oligonucleotide probes, which correspond to a part of the sequence encoding the protein in question, can be synthesized chemically. This requires that short, oligopeptide stretches of amino acid sequence must be known. The DNA sequence encoding the protein can be deduced from the genetic code, however, the degeneracy of the code must be taken into account. It is possible to perform a mixed addition reaction when the sequence is degenerate. This includes a heterogeneous mixture of denatured double-stranded DNA. For such screening, hybridization is preferably performed on either single-stranded DNA or denatured double-stranded DNA. Hybridization is particularly useful in the detection of DNA clones derived from sources where an extremely low amount of mRNA sequences relating to the polypeptide of interest are present. In other words, by using stringent hybridization conditions directed to avoid non-specific binding, it is possible, for example, to allow the autoradiographic visualization of a specific cDNA clone by the hybridization of the target DNA to that single probe in the mixture which is its complete complement (Wallace, *et al.*, Nucl. Acid Res., 9:879, 1981).

[0097] When the entire sequence of amino acid residues of the desired polypeptide is not known, the direct synthesis of DNA sequences is not possible and the method for choice is use of cDNA sequences. Among the standard procedures for isolating cDNA sequences of interest is the formation of plasmid- or phage-carrying cDNA libraries, which are derived from reverse transcription of mRNA, which is abundant in donor cells

that have a high level of genetic expression. When used in combination with polymerase chain reaction technology, even rare expression products can be cloned.

[0098] DNA sequences encoding an invention ANKTM1-related polypeptide can be expressed *in vitro* by DNA transfer into a suitable host cell, as described above.

[0099] The invention ANKTM1-related polynucleotide sequences may be inserted into a recombinant expression vector. The term "recombinant expression vector" refers to a plasmid, virus or other vehicle known in the art that has been manipulated by insertion or incorporation of the nociception-related genetic sequences. Such expression vectors contain a promoter sequence that facilitates the efficient transcription of the inserted genetic sequence of the host. The expression vector typically contains an origin of replication, a promoter, as well as specific genes that allow phenotypic selection of the transformed cells. Vectors suitable for use in the present invention include those described above.

[0100] Polynucleotide sequences encoding a nociception-related polypeptide can be expressed in either prokaryotes or eukaryotes. Hosts can include microbial, yeast, insect and mammalian organisms. Such vectors are used to incorporate DNA sequences of the invention.

[0101] Methods that are well known to those skilled in the art can be used to construct expression vectors containing the ANKTM1-related polypeptide coding sequence and appropriate transcriptional/translational control signals. These methods include *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* recombination/genetic techniques. (See, for example, the techniques described in Maniatis *et al.*, 1989, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y.).

[0102] The genetic construct can be designed to provide additional benefits, such as, for example addition of C-terminal or N-terminal amino acid residues that would facilitate purification by trapping on columns or by use of antibodies. All those methodologies are cumulative. For example, a synthetic gene can later be mutagenized.

The choice as to the method for producing a particular construct can easily be made by one skilled in the art based on practical considerations: size of the desired peptide, availability and cost of starting materials, etc. All the technologies involved are well established and well known in the art. See, for example, Ausubel *et al.*, Current Protocols in Molecular Biology, Volumes 1 and 2 (1987), with supplements, and Maniatis *et al.*, Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory (1989). Yet other technical references are known and easily accessible to one skilled in the art.

[0103] In another embodiment, the invention provides antibodies that bind to an invention ANKTM1-related polypeptide. Such antibodies are useful for research and diagnostics in the study of pain, nociceptive responses, central nervous system regulation and modulation of pain, and nociceptive-associated pathologies in general. For example, the invention allows for the diagnosis in a subject of hyperalgesia associated with improper nociceptive pain regulation. Preferably the subject is a human.

[0104] Such antibodies may be administered alone or contained in a pharmaceutical composition comprising antibodies against an invention ANKTM1-related polypeptide and other reagents effective as modulators of nociceptive pain and associated pain disorders both *in vitro* and *in vivo*.

[0105] The term "epitope", as used herein, refers to an antigenic determinant on an antigen, such as an invention ANKTM1-related polypeptide, to which the paratope of an antibody, such as an antibody that binds to an invention ANKTM1-related polypeptide. Antigenic determinants usually consist of chemically active surface groupings of molecules, such as amino acids or sugar side chains, and can have specific three-dimensional structural characteristics, as well as specific charge characteristics.

[0106] Antibodies that bind to a polypeptide of the invention can be prepared using an intact polypeptide or fragments containing small peptides of interest as the immunizing antigen. The polypeptide or a peptide used to immunize an animal can be derived from translated cDNA or chemical synthesis, which can be conjugated to a carrier protein, if

desired. Such commonly used carriers, which are chemically coupled to the peptide, include keyhole limpet hemocyanin (KLH), thyroglobulin, bovine serum albumin (BSA), and tetanus toxoid. The coupled peptide is then used to immunize the animal (*e.g.*, a mouse, a rat, or a rabbit).

[0107] If desired, polyclonal or monoclonal antibodies can be further purified, for example, by binding to and elution from a matrix to which the polypeptide or a peptide to which the antibodies were raised is bound. Those of skill in the art will know of various techniques common in the immunology arts for purification and/or concentration of polyclonal antibodies, as well as monoclonal antibodies (See for example, Coligan, *et al.*, Unit 9, Current Protocols in Immunology, Wiley Interscience, 1991, incorporated by reference).

[0108] It is also possible to use the anti-idiotypic technology to produce monoclonal antibodies that mimic an epitope. For example, an anti-idiotypic monoclonal antibody made to a first monoclonal antibody will have a binding domain in the hypervariable region that is the "image" of the epitope bound by the first monoclonal antibody.

[0109] Antibodies of the invention include polyclonal antibodies, monoclonal antibodies, and fragments of polyclonal and monoclonal antibodies.

[0110] The preparation of polyclonal antibodies is well known to those skilled in the art. See, for example, Green *et al.*, Production of Polyclonal Antisera, in Immunochemical Protocols (Manson, ed.), pages 1-5 (Humana Press 1992); Coligan *et al.*, Production of Polyclonal Antisera in Rabbits, Rats, Mice and Hamsters, in Current Protocols in Immunology, section 2.4.1 (1992), which are hereby incorporated by reference.

[0111] The preparation of monoclonal antibodies likewise is conventional. See, for example, Kohler & Milstein, *Nature*, 256:495 (1975); Coligan *et al.*, sections 2.5.1-2.6.7; and Harlow *et al.*, Antibodies: A Laboratory Manual, page 726 (Cold Spring Harbor Pub. 1988), which are hereby incorporated by reference. Briefly, monoclonal antibodies can

be obtained by injecting mice with a composition comprising an antigen, verifying the presence of antibody production by removing a serum sample, removing the spleen to obtain B lymphocytes, fusing the B lymphocytes with myeloma cells to produce hybridomas, cloning the hybridomas, selecting positive clones that produce antibodies to the antigen, and isolating the antibodies from the hybridoma cultures. Monoclonal antibodies can be isolated and purified from hybridoma cultures by a variety of well-established techniques. Such isolation techniques include affinity chromatography with Protein-A Sepharose, size-exclusion chromatography, and ion-exchange chromatography. See, *e.g.*, Coligan *et al.*, sections 2.7.1-2.7.12 and sections 2.9.1-2.9.3; Barnes *et al.*, Purification of Immunoglobulin G (IgG), in *Methods in Molecular Biology*, Vol. 10, pages 79-104 (Humana Press 1992). Methods for *in vitro* and *in vivo* multiplication of monoclonal antibodies is well known to those skilled in the art. Multiplication *in vitro* may be carried out in suitable culture media such as Dulbecco's Modified Eagle Medium or RPMI 1640 medium, optionally replenished by a mammalian serum such as fetal calf serum or trace elements and growth-sustaining supplements such as normal mouse peritoneal exudate cells, spleen cells, bone marrow macrophages. Production *in vitro* provides relatively pure antibody preparations and allows scale-up to yield large amounts of the desired antibodies. Large-scale hybridoma cultivation can be carried out by homogenous suspension culture in an airlift reactor, in a continuous stirrer reactor, or in immobilized or entrapped cell culture. Multiplication *in vivo* may be carried out by injecting cell clones into mammals histocompatible with the parent cells, *e.g.*, syngeneic mice, to cause growth of antibody-producing tumors. Optionally, the animals are primed with a hydrocarbon, especially oils such as pristane tetramethylpentadecane prior to injection. After one to three weeks, the desired monoclonal antibody is recovered from the body fluid of the animal.

[0112] Therapeutic applications for antibodies disclosed herein are also part of the present invention. For example, antibodies of the present invention may also be derived from subhuman primate antibody. General techniques for raising therapeutically useful antibodies in baboons can be found, for example, in Goldenberg *et al.*, International

Patent Publication WO 91/11465 (1991) and Losman *et al.*, *Int. J. Cancer*, 46:310 (1990), which are hereby incorporated by reference.

[0113] Alternatively, an anti-ANKTM1-related polypeptide antibody may be derived from a "humanized" monoclonal antibody. Humanized monoclonal antibodies are produced by transferring mouse complementarity determining regions from heavy and light variable chains of the mouse immunoglobulin into a human variable domain, and then substituting human residues in the framework regions of the murine counterparts. The use of antibody components derived from humanized monoclonal antibodies obviates potential problems associated with the immunogenicity of murine constant regions. General techniques for cloning murine immunoglobulin variable domains are described, for example, by Orlandi *et al.*, *Proc. Nat'l Acad. Sci. USA*, 86:3833 (1989), which is hereby incorporated in its entirety by reference. Techniques for producing humanized monoclonal antibodies are described, for example, by Jones *et al.*, *Nature*, 321:522 (1986); Riechmann *et al.*, *Nature*, 332:323 (1988); Verhoeyen *et al.*, *Science*, 239:1534 (1988); Carter *et al.*, *Proc. Nat'l Acad. Sci. USA*, 89:4285 (1992); Sandhu, *Crit. Rev. Biotech.*, 12:437 (1992); and Singer *et al.*, *J. Immunol.*, 150:2844 (1993), which are hereby incorporated by reference.

[0114] Antibodies of the invention also may be derived from human antibody fragments isolated from a combinatorial immunoglobulin library. See, for example, Barbas *et al.*, *Methods: A Companion to Methods in Enzymology*, Vol. 2, page 119 (1991); Winter *et al.*, *Ann. Rev. Immunol.* 12:433 (1994), which are hereby incorporated by reference. Cloning and expression vectors that are useful for producing a human immunoglobulin phage library can be obtained, for example, from Stratagene Cloning Systems (La Jolla, CA).

[0115] In addition, antibodies of the present invention may be derived from a human monoclonal antibody. Such antibodies are obtained from transgenic mice that have been "engineered" to produce specific human antibodies in response to antigenic challenge. In this technique, elements of the human heavy and light chain loci are introduced into strains of mice derived from embryonic stem cell lines that contain targeted disruptions of

the endogenous heavy and light chain loci. The transgenic mice can synthesize human antibodies specific for human antigens, and the mice can be used to produce human antibody-secreting hybridomas. Methods for obtaining human antibodies from transgenic mice are described by Green *et al.*, *Nature Genet.*, 7:13 (1994); Lonberg *et al.*, *Nature*, 368:856 (1994); and Taylor *et al.*, *Int. Immunol.*, 6:579 (1994), which are hereby incorporated by reference.

[0116] Antibody fragments of the invention can be prepared by proteolytic hydrolysis of the antibody or by expression in *E. coli* of DNA encoding the fragment. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. For example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted F(ab')₂. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulfhydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab' fragments and an Fc fragment directly. These methods are described, for example, by Goldenberg in U.S. patents No. 4,036,945 and No. 4,331,647, and references contained therein. These patents are hereby incorporated in their entireties by reference. See also Nisonhoff *et al.*, *Arch. Biochem. Biophys.*, 89:230 (1960); Porter, *Biochem. J.*, 73:119 (1959); Edelman *et al.*, *Methods in Enzymology*, Vol. 1, page 422 (Academic Press 1967); and Coligan *et al.* at sections 2.8.1-2.8.10 and 2.10.1-2.10.4.

[0117] Other methods for cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

[0118] For example, Fv fragments comprise an association of V_H and V_L chains. This association may be noncovalent, as described in Inbar *et al.*, *Proc. Nat'l Acad. Sci. USA*, 69:2659 (1972). Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde. See, *e.g.*, Sandhu,

supra. Preferably, the F_v fragments comprise V_H and V_L chains connected by a peptide linker. These single-chain antigen binding proteins (sFv) are prepared by constructing a structural gene comprising DNA sequences encoding the V_H and V_L domains connected by an oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing sFvs are described, for example, by Whitlow *et al.*, Methods: A Companion to Methods in Enzymology, Vol. 2, page 97 (1991); Bird *et al.*, Science, 242:423 (1988); Ladner *et al.*, U.S. patent No. 4,946,778; Pack *et al.*, Bio/Technology, 11:1271 (1993); and Sandhu, *supra*.

[0119] Another form of an antibody fragment is a peptide coding for a single complementarity- determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells. See, for example, Larrick *et al.*, Methods: A Companion to Methods in Enzymology, Vol. 2, page 106 (1991).

[0120] In one embodiment, the invention provides a method for modulating (*e.g.*, inhibiting) nociceptive pain in a subject by administering to a cell or subject an effective amount of a composition which contains an invention ANKTM1-related polypeptide, or biologically functional fragment thereof, or an agent (*e.g.*, an antibody, ribozyme, antisense molecule, or double-stranded interfering RNA molecules) that interacts with or inhibits expression of an invention ANKTM1-related polypeptide.

[0121] As used herein, an "effective amount" of a composition containing ANKTM1-related polypeptide or an invention ANKTM1-related polypeptide -modulating agent is defined as that amount that is effective in modulating nociceptive pain or a nociceptive response in a subject. For example, an inhibitory-effective amount would be that amount of the composition or agent sufficient to inhibit a nociceptive pain response. One skilled in the art can easily identify agents that modulate as well as the effective amount of an

agent that modulates nociceptive response by using, for example, the intracellular calcium imaging tests described in the Examples herein and others as are known in the art.

Briefly, a determination can be made as to the effectiveness or effective concentration or amount of an agent by contacting a cell expressing an invention ANKTM1-related polypeptide, such as mANKTM1 or hANKTM1 with the test agent or concentration and then exposing the cell to a noxious agent (*e.g.*, a temperature of about 10-15 °C) and determining the cell's response (*e.g.*, using electrophysiological studies or intracellular calcium imaging techniques as described herein) in the presence and absence of the agent.

[0122] In another embodiment, the present invention provides a method for modulating expression of an invention ANKTM1-related polypeptide as well as methods for screening for agents that modulate ANKTM1-related polypeptide gene expression. In this embodiment, a cell or subject is contacted with an agent suspected or known to have ANKTM1-related polypeptide expression modulating activity. The change in ANKTM1-related polypeptide gene expression is then measured as compared to a control or standard sample. The control or standard sample can be the baseline expression of the cell or subject prior to contact with the agent. An agent that modulates ANKTM1-related polypeptide gene expression may be a polynucleotide, for example, the polynucleotide may be an antisense, a triplex agent, a ribozyme, or a double-stranded interfering RNA. For example, an antisense molecule may be directed to the structural gene region or to the promoter region of ANKTM1-related polypeptide gene. The agent may be an agonist, antagonist, peptide, peptidomimetic, antibody, or chemical.

[0123] Double-stranded interfering RNA molecules are especially useful to inhibit expression of a target gene. For example, double-stranded RNA molecules can be injected into a target cell or organism to inhibit expression of a gene and the resultant gene products activity. It has been found that such double-stranded RNA molecules are more effective at inhibiting expression than either RNA strand alone. (Fire *et al.*, Nature, 1998, 19:391(6669):806-11).

[0124] When a disorder is associated with abnormal expression of an invention ANKTM1-related polypeptide (*e.g.*, overexpression, or expression of a mutated form of

the protein), a therapeutic approach that directly interferes with the translation of an invention ANKTM1-related polypeptide is possible. Alternatively, similar methodology may be used to study gene activity. For example, antisense nucleic acid, double-stranded interfering RNA or ribozymes could be used to bind to the invention ANKTM1-related polypeptide mRNA sequence or to cleave it. Antisense RNA or DNA molecules bind specifically with a targeted gene's RNA message, interrupting the expression of that gene's protein product. The antisense binds to the messenger RNA forming a double stranded molecule that cannot be translated by the cell. Antisense oligonucleotides of about 15-25 nucleotides are preferred since they are easily synthesized and have an inhibitory effect just like antisense RNA molecules. In addition, chemically reactive groups, such as iron-linked ethylenediaminetetraacetic acid (EDTA-Fe) can be attached to an antisense oligonucleotide, causing cleavage of the RNA at the site of hybridization. Antisense nucleic acids are DNA or RNA molecules that are complementary to at least a portion of a specific mRNA molecule (Weintraub, Scientific American, 262:40, 1990). In the cell, the antisense nucleic acids hybridize to the corresponding mRNA, forming a double-stranded molecule. The antisense nucleic acids interfere with the translation of the mRNA, since the cell will not translate an mRNA that is double-stranded. Antisense oligomers of about 15 nucleotides are preferred, since they are easily synthesized and are less likely to cause problems than larger molecules when introduced into the target ANKTM1-related polypeptide-producing cell. The use of antisense methods to inhibit the *in vitro* translation of genes is well known in the art (Marcus-Sakura, Anal. Biochem., 172:289, 1988).

[0125] Use of an oligonucleotide to stall transcription is known as the triplex strategy since the oligomer winds around double-helical DNA, forming a three-strand helix. Therefore, these triplex compounds can be designed to recognize a unique site on a chosen gene (Maher, *et al.*, Antisense Res. and Dev., 1:227, 1991; Helene, Anticancer Drug Design, 6:569, 1991).

[0126] Ribozymes are RNA molecules possessing the ability to specifically cleave other single-stranded RNA in a manner analogous to DNA restriction endonucleases.

Through the modification of nucleotide sequences that encode these RNAs, it is possible to engineer molecules that recognize specific nucleotide sequences in an RNA molecule and cleave it (Cech, J.Amer.Med. Assn., 260:3030, 1988). A major advantage of this approach is that, because they are sequence-specific, only mRNAs with particular sequences are inactivated.

[0127] There are two basic types of ribozymes namely, tetrahymena-type (Hasselhoff, Nature, 334:585, 1988) and "hammerhead"-type. Tetrahymena-type ribozymes recognize sequences that are four bases in length, while "hammerhead"-type ribozymes recognize base sequences 11-18 bases in length. The longer the recognition sequence, the greater the likelihood that the sequence will occur exclusively in the target mRNA species. Consequently, hammerhead-type ribozymes are preferable to tetrahymena-type ribozymes for inactivating a specific mRNA species and 18-base recognition sequences are preferable to shorter recognition sequences.

[0128] These and other uses of antisense and ribozymes methods to inhibit the *in vivo* translation of genes are known in the art (e.g., De Mesmaeker, *et al.*, Curr. Opin. Struct. Biol., 5:343, 1995; Gewirtz, A.M., *et al.*, Proc. Natl. Acad. Sci. U.S.A., 93:3161, 1996b; Stein, C.A., Chem. and Biol. 3:319, 1996).

[0129] Delivery of antisense, triplex agents, ribozymes, competitive inhibitors, double-stranded interfering RNA and the like can be achieved using a recombinant expression vector such as a chimeric virus or a colloidal dispersion system or by injection. Various viral vectors which can be utilized for gene therapy as taught herein include adenovirus, herpes virus, vaccinia, or, preferably, an RNA virus such as a retrovirus. Preferably, the retroviral vector is a derivative of a murine or avian retrovirus. Examples of retroviral vectors in which a single foreign gene can be inserted include, but are not limited to: Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), and Rous Sarcoma Virus (RSV). A number of additional retroviral vectors can incorporate multiple genes. All of these vectors can transfer or incorporate a gene for a selectable marker so that transduced cells can be identified and generated. By inserting a polynucleotide sequence of interest

into the viral vector, along with another gene that encodes the ligand for a receptor on a specific target cell, for example, the vector is now target specific. Retroviral vectors can be made target specific by inserting, for example, a polynucleotide encoding a sugar, a glycolipid, or a protein. Preferred targeting is accomplished by using an antibody to target the retroviral vector. Those of skill in the art will know of, or can readily ascertain without undue experimentation, specific polynucleotide sequences which can be inserted into the retroviral genome to allow target specific delivery of the retroviral vector containing the antisense polynucleotide.

[0130] Another targeted delivery system for polynucleotides is a colloidal dispersion system. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. The preferred colloidal system of this invention is a liposome. Liposomes are artificial membrane vesicles that are useful as delivery vehicles *in vitro* and *in vivo*. It has been shown that large unilamellar vesicles (LUV), which range in size from 0.2-4.0 μm can encapsulate a substantial percentage of an aqueous buffer containing large macromolecules. RNA, DNA and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley, *et al.*, Trends Biochem. Sci., 6:77, 1981). In addition to mammalian cells, liposomes have been used for delivery of polynucleotides in plant, yeast and bacterial cells. In order for a liposome to be an efficient gene transfer vehicle, the following characteristics should be present: (1) encapsulation of the genes of interest at high efficiency while not compromising their biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information (Mannino, *et al.*, Biotechniques, 6:682, 1988).

[0131] The composition of the liposome is usually a combination of phospholipids, particularly high-phase-transition-temperature phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used.

The physical characteristics of liposomes depend on pH, ionic strength, and the presence of divalent cations.

[0132] Examples of lipids useful in liposome production include phosphatidyl compounds, such as phosphatidylglycerol, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingolipids, cerebrosides, and gangliosides. Particularly useful are diacylphosphatidyl-glycerols, where the lipid moiety contains from 14-18 carbon atoms, particularly from 16-18 carbon atoms, and is saturated. Illustrative phospholipids include egg phosphatidylcholine, dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine.

[0133] The targeting of liposomes has been classified based on anatomical and mechanistic factors. Anatomical classification is based on the level of selectivity, for example, organ-specific, cell-specific, and organelle-specific. Mechanistic targeting can be distinguished based upon whether it is passive or active. Passive targeting utilizes the natural tendency of liposomes to distribute to cells of the reticulo-endothelial system (RES) in organs that contain sinusoidal capillaries. Active targeting, on the other hand, involves alteration of the liposome by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein, or by changing the composition or size of the liposome in order to achieve targeting to organs and cell types other than the naturally occurring sites of localization.

[0134] The surface of the targeted delivery system may be modified in a variety of ways. In the case of a liposomal targeted delivery system, lipid groups can be incorporated into the lipid bilayer of the liposome in order to maintain the targeting ligand in stable association with the liposomal bilayer. Various linking groups can be used for joining the lipid chains to the targeting ligand. In general, the compounds bound to the surface of the targeted delivery system will be ligands and receptors which will allow the targeted delivery system to find and "home in" on the desired cells. A ligand may be any compound of interest that will bind to another compound, such as a receptor.

[0135] The agents useful in the method for the invention can be administered, for in vivo application, parenterally by injection or by gradual perfusion over time. Administration may be intravenously, intraperitoneally, intramuscularly, subcutaneously, intracavity, or transdermally. For *in vitro* studies the agents may be added or dissolved in an appropriate biologically acceptable buffer and added to a cell or tissue.

[0136] Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents and inert gases and the like.

[0137] It is envisioned that the invention can be used to treat pathologies associated with hyperalgesia and nociceptive pain associated disorders. Therefore, the present invention encompasses methods for ameliorating a disorder associated with nociception, including treating a subject having the disorder, at the site of the disorder, with an agent which modulates an invention ANKTM1-related polypeptide. Generally, the terms "treating", "treatment" and the like are used herein to mean affecting a subject, tissue or cell to obtain a desired pharmacologic and/or physiologic effect. The effect may be prophylactic in terms of completely or partially preventing a disease or sign or symptom thereof, and/or may be therapeutic in terms of a partial or complete cure for hyperalgesia and nociceptive pain associated disorders and/or adverse effect, such as pain, attributable to the hyperalgesia and nociceptive pain associated disorders. "Treating" as used herein covers any treatment of, or prevention of hyperalgesia and nociceptive pain associated disorders in an invertebrate, a vertebrate, a mammal, particularly a human, and includes: (a) preventing the disorder from occurring in a subject that may be predisposed to the

disorder, but has not yet been diagnosed as having it; (b) inhibiting the disorder, *i.e.*, arresting its development; or (c) relieving or ameliorating the disorder cause regression of one or more symptoms of the hyperalgesia and/or nociceptive pain associated disorder.

[0138] The invention includes various pharmaceutical compositions useful for ameliorating symptoms attributable to a nociceptive pain-associated disorder. The pharmaceutical compositions according to the invention are prepared by bringing an antibody against ANKTM1-related polypeptide, a polypeptide or peptide derivative of an invention ANKTM1-related polypeptide, an invention ANKTM1-related polypeptide mimetic, a drug, chemical or combination of chemicals or an invention ANKTM1-related polypeptide-modulating agent into a form suitable for administration to a subject using carriers, excipients and additives or auxiliaries. Frequently used carriers or auxiliaries include magnesium carbonate, titanium dioxide, lactose, mannitol and other sugars, talc, milk protein, gelatin, starch, vitamins, cellulose and its derivatives, animal and vegetable oils, polyethylene glycols and solvents, such as sterile water, alcohols, glycerol and polyhydric alcohols. Intravenous vehicles include fluid and nutrient replenishers. Preservatives include antimicrobial, anti-oxidants, chelating agents and inert gases. Other pharmaceutically acceptable carriers include aqueous solutions, non-toxic excipients, including salts, preservatives, buffers and the like, as described, for instance, in Remington's Pharmaceutical Sciences, 15th ed. Easton: Mack Publishing Co., 1405-1412, 1461-1487 (1975) and The National Formulary XIV., 14th ed. Washington: American Pharmaceutical Association (1975), the contents of which are hereby incorporated by reference. The pH and exact concentration of the various components of the pharmaceutical composition are adjusted according to routine skills in the art. See Goodman and Gilman's The Pharmacological Basis for Therapeutics (7th ed.).

[0139] The pharmaceutical compositions are preferably prepared and administered in dose units. Solid dose units are tablets, capsules and suppositories. For treatment of a subject, depending on activity of the compound, manner of administration, nature and severity of the disorder, age and body weight of the subject, different daily doses are necessary. Under certain circumstances, however, higher or lower daily doses may be

appropriate. The administration of the daily dose can be carried out both by single administration in the form of an individual dose unit or else several smaller dose units and also by multiple administrations of subdivided doses at specific intervals.

[0140] The pharmaceutical compositions according to the invention may be administered locally or systemically in a therapeutically effective dose. Amounts effective for this use will, of course, depend on the severity of the disease and the weight and general state of the subject. Typically, dosages used *in vitro* may provide useful guidance in the amounts useful for *in situ* administration of the pharmaceutical composition, and animal models may be used to determine effective dosages for treatment of particular disorders. Various considerations are described, *e.g.*, in Langer, Science, 249:1527, (1990); Gilman *et al.* (eds.) (1990), each of which is herein incorporated by reference.

[0141] In one embodiment, the invention provides a pharmaceutical composition useful for administering an invention ANKTM1-related polypeptide, or nucleic acid encoding an invention ANKTM1-related polypeptide, to a subject in need of such treatment. "Administering" the pharmaceutical composition of the present invention may be accomplished by any means known to the skilled artisan. Preferably a "subject" refers to a mammal, most preferably a human, but may be any organism.

[0142] An invention ANKTM1-related polypeptide or antibody can be administered parenterally, enterically, by injection, rapid infusion, nasopharyngeal absorption, dermal absorption, rectally and orally. Pharmaceutically acceptable carrier preparations for parenteral administration include sterile or aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Carriers for occlusive dressings can be used to increase skin permeability and enhance antigen absorption. Liquid dosage forms for oral administration may generally comprise a liposome solution containing the liquid dosage form. Suitable solid or liquid pharmaceutical preparation forms are, for example, granules, powders, tablets, coated tablets, (micro)capsules, suppositories, syrups, emulsions, suspensions, creams, aerosols, drops or injectable solution in ampule form and also preparations with

protracted release of active compounds, in whose preparation excipients and additives and/or auxiliaries such as disintegrants, binders, coating agents, swelling agents, lubricants, flavorings, sweeteners and elixirs containing inert diluents commonly used in the art, such as purified water.

[0143] In another embodiment, the invention provides a method for identifying a agent which modulates ANKTM1-related polypeptide expression or activity including incubating components comprising the agent and an invention ANKTM1-related polypeptide, or a recombinant cell expressing an invention ANKTM1-related polypeptide, under conditions sufficient to allow the agent to interact and determining the affect of the agent on the expression or activity of the gene or polypeptide, respectively. The term "affect", as used herein, encompasses any means by which gene expression or protein activity can be modulated. Such agents can include, for example, polypeptides, peptidomimetics, chemical compounds, small molecules and biologic agents as described below.

[0144] Incubating includes conditions that allow contact between the test agent and an invention ANKTM1-related polypeptide, a cell expressing an invention ANKTM1-related polypeptide or nucleic acid encoding an invention ANKTM1-related polypeptide. Contacting includes in solution and in solid phase. The test agent may optionally be a combinatorial library for screening a plurality of agents. Agents identified in the method for the invention can be further evaluated, detected, cloned, sequenced, and the like, either in solution or after binding to a solid support, by any method usually applied to the detection of a specific DNA sequence such as PCR, oligomer restriction (Saiki, *et al.*, Bio/Technology, 3:1008-1012, 1985), oligonucleotide ligation assays (OLAs) (Landegren, *et al.*, Science, 241:1077, 1988), and the like. Molecular techniques for DNA analysis have been reviewed (Landegren, *et al.*, Science, 242:229-237, 1988).

[0145] Thus, the method for the invention includes combinatorial chemistry methods for identifying chemical agents that bind to or affect ANKTM1-related polypeptide expression or activity.

[0146] Areas of investigation are the development of therapeutic treatments. The screening identifies agents that provide modulation of ANKTM1-related polypeptide function in targeted organisms. Of particular interest are screening assays for agents that have a low toxicity or a reduced number of side effects for humans.

[0147] The term "agent" as used herein describes any molecule, *e.g.* protein or pharmaceutical, with the capability of altering or mimicking the physiological function or expression of an invention ANKTM1-related polypeptide. Generally, a plurality of assay mixtures is run in parallel with different agent concentrations to obtain a differential response to the various concentrations. Typically, one of these concentrations serves as a negative control, *i.e.* at zero concentration or below the level of detection.

[0148] As used herein, an agent that acts, directly or indirectly via a receptor or receptors responsible for mediating or involved in peripheral hyperalgesia, by antagonizing the activity of hyperalgesia mediating agents, such as a prostaglandin, is an agent intended for use herein, if it also does not exhibit CNS effects as defined herein. Such agent is a peripheral antihyperalgesic. The activity of antihyperalgesic agents is distinct from the activity of centrally acting analgesic agents (*e.g.*, agents that act by virtue of crossing the blood brain barrier). Anti-hyperalgesic agents act to block hypersensitivity. The compositions and methods for the invention are intended for prevention and/or the amelioration of the symptoms of hyperalgesia by decreasing or eliminating the hyperalgesia or by preventing its onset. An antihyperalgesic agent is distinct from a local anesthetic, which is an agent that produces numbness by abolishing sensitivity to touch, and other stimuli, including pain stimuli. Local anesthetics abolish sensation, including pain, by blocking conduction in nerve axons in the peripheral nervous system. Antihyperalgesics, on the other hand, alleviate pain by elevating a patient's threshold to pain. Thus, unlike anesthetics, antihyperalgesics reduce sensation to pain during states of increased sensitivity (*e.g.*, hyperalgesia) without substantially affecting normal sensitivity to touch and/or other stimuli.

[0149] Antihyperalgesics are agents that may reduce hypersensitivity to touch and other stimuli that would not, under normal circumstances, evoke a pain response. The

hyperalgesic response is an exaggerated response, such as excessive sensitiveness or sensibility to pain from touch, slight exertion, warmth and the like. Antihyperalgesics may be identified, for example, by the Randall-Selitto method (*see, e.g.,* Randall *et al.* Arch. Int. Pharmacodyn. 111:409-419, 1957), as well as the formalin, carrageenan and yeast induced inflammation methods. In addition to the antihyperalgesic effect, the antihyperalgesic agents provided herein may concurrently provide an analgesic effect.

[0150] Analgesics are agents that may reduce a patient's perception of pain evoked by stimuli that are acutely painful under normal circumstances. Thus, analgesics may be effective in reducing the acute and immediate pain associated with trauma (*e.g.,* pinpricks, burns, or crushing wounds) as well as chronic pain, that is not normally associated with peripheral sensitization, such as cancer or headache pain.

[0151] In addition, cells or organisms which have a mutation in an invention ANKTM1-related polypeptide sequence may be used as models to screen for agents which modulate disorders associated with the mutation. For example, if organisms are identified that lack normal nociceptive response activity due to a mutation in an invention nociception-related polypeptide sequence, administration of agents to an organism having such a mutation, or cells derived or recombinantly modified to have a reduced nociceptive activity, may be used to determine the effect of the drug or agent on nociception.

[0152] In a further embodiment, the invention provides a method for detecting ANKTM1-related polypeptide or polynucleotide or diagnosing a nociceptive-associated disorder in a subject including contacting a cell component containing ANKTM1-related polypeptide or polynucleotide with a reagent which binds to the cell polypeptide or polynucleotide (herein after cell component). The cell component can be or contain a nucleic acid, such as DNA or RNA, or a protein. When the component is nucleic acid, the reagent is a nucleic acid probe or PCR primer. When the cell component is protein, the reagent is an antibody probe. The probes are detectably labeled, for example, with a radioisotope, a fluorescent compound, a bioluminescent compound, a chemiluminescent compound, a metal chelator or an enzyme. Those of ordinary skill in the art will know of

other labels suitable for binding to an antibody or nucleic acid probe, or will be able to ascertain such, using routine experimentation. There are many different labels and methods for labeling known to those of ordinary skill in the art. Examples of the types of labels, which can be used in the present invention, include enzymes, radioisotopes, colloidal metals, fluorescent compounds, chemiluminescent compounds, and bioluminescent compounds. In addition, the antibodies, polypeptides and polynucleotide sequences of the invention can be used to diagnosis a nociceptive disorder.

[0153] A monoclonal antibody of the invention, directed toward ANKTM1-related polypeptide is useful for the *in vivo* and *in vitro* detection of antigen. The detectably labeled monoclonal antibody is given in a dose that is diagnostically effective. The term "diagnostically effective" means that the amount of detectably labeled monoclonal antibody is administered in sufficient quantity to enable detection of ANKTM1-related polypeptide antigen for which the monoclonal antibodies are specific.

[0154] The concentration of a detectably labeled monoclonal antibody administered to a subject should be sufficient such that the binding to those cells, body fluid, or tissue having ANKTM1-related polypeptide that is detectable compared to the background. Further, it is desirable that the detectably labeled monoclonal antibody be rapidly cleared from the circulatory system in order to give the best target-to-background signal ratio.

[0155] For *in vivo* diagnostic imaging, the type of detection instrument available is a major factor in selecting a given radioisotope. The radioisotope chosen must have a type of decay that is detectable for a given type of instrument. Still another important factor in selecting a radioisotope for *in vivo* diagnosis is the half-life of the radioisotope which should be long enough so that it is still detectable at the time of maximum uptake by the target, but short enough so that deleterious radiation with respect to the host is minimized. Ideally, a radioisotope used for *in vivo* imaging will lack a particle emission, but produce a large number of photons in the 140-250 nm key range, which may be readily detected by conventional gamma cameras.

[0156] For *in vivo* diagnosis, radioisotopes may be bound to immunoglobulin either directly or indirectly by using an intermediate functional group. Intermediate functional groups, which often are used to bind radioisotopes that exist as metallic ions to immunoglobulins, are the bifunctional chelating agents such as diethylenetriaminepentacetic acid (DTPA) and ethylenediaminetetraacetic acid (EDTA) and similar molecules. Typical examples of metallic ions that can be bound to the monoclonal antibodies of the invention are ^{111}In , ^{97}Ru , ^{67}Ga , ^{68}Ga , ^{72}As , ^{89}Zr , and ^{201}Tl .

[0157] The monoclonal antibodies of the invention can also be labeled with a paramagnetic isotope for purposes of *in vivo* diagnosis, as in magnetic resonance imaging (MRI) or electron spin resonance (ESR). In general, any conventional method for visualizing diagnostic imaging can be utilized. Usually gamma and positron emitting radioisotopes are used for camera imaging and paramagnetic isotopes for MRI. Elements that are particularly useful in such techniques include ^{157}Gd , ^{55}Mn , ^{162}Dy , ^{52}Cr , and ^{56}Fe .

[0158] In another embodiment, nucleic acid probes can be used to identify a polynucleotide encoding an invention ANKTM1-related polypeptide from a specimen obtained from a subject. Examples of specimens from which nucleic acid sequence encoding an invention ANKTM1-related polypeptide can be derived include insect, human, swine, porcine, feline, canine, equine, murine, cervine, caprine, lupine, leporidine and bovine species.

[0159] Oligonucleotide probes, which correspond to a part of the sequence encoding the protein in question, can be synthesized chemically. This requires that short, oligopeptide stretches of amino acid sequence must be known. The DNA sequence encoding the protein can be deduced from the genetic code, however, the degeneracy of the code must be taken into account. It is possible to perform a mixed addition reaction when the sequence is degenerate. This includes a heterogeneous mixture of denatured double-stranded DNA. For such screening, hybridization is preferably performed on either single-stranded DNA or denatured double-stranded DNA. Hybridization is particularly useful in the detection of cDNA clones derived from sources where an extremely low amount of mRNA sequences relating to the polypeptide of interest are

present. In other words, by using stringent hybridization conditions directed to avoid non-specific binding, it is possible, for example, to allow the autoradiographic visualization of a specific cDNA clone by the hybridization of the target DNA to that single probe in the mixture which is its complete complement (Wallace, *et al.*, Nucl. Acid Res. 9:879, 1981).

[0160] In an embodiment of the invention, purified nucleic acid fragments containing intervening sequences or oligonucleotide sequences of 10-50 base pairs are radioactively labeled. The labeled preparations are used to probe nucleic acids from a specimen by the Southern hybridization technique. Nucleotide fragments from a specimen, before or after amplification, are separated into fragments of different molecular masses by gel electrophoresis and transferred to filters that bind nucleic acid. After exposure to the labeled probe, which will hybridize to nucleotide fragments containing target nucleic acid sequences, binding of the radioactive probe to target nucleic acid fragments is identified by autoradiography (see Genetic Engineering, 1, ed. Robert Williamson, Academic Press, (1981), 72-81). Alternatively, nucleic acid from the specimen can be bound directly to filters to which the radioactive probe selectively attaches by binding nucleic acids having the sequence of interest. Specific sequences and the degree of binding are quantitated by directly counting the radioactive emissions.

[0161] Where the target nucleic acid is not amplified, detection using an appropriate hybridization probe may be performed directly on the separated nucleic acid. In those instances where the target nucleic acid is amplified, detection with the appropriate hybridization probe would be performed after amplification.

[0162] For the most part, the probe will be detectably labeled with an atom or inorganic radical, most commonly using radionuclides, but also heavy metals can be used. Conveniently, a radioactive label may be employed. Radioactive labels include ^{32}P , ^{125}I , ^3H , ^{14}C , ^{111}In , ^{99}Tc , or the like. Any radioactive label may be employed which provides for an adequate signal and has sufficient half-life. Other labels include ligands, which can serve as a specific binding pair member for a labeled ligand, and the like. A wide variety of labels routinely employed in immunoassays can readily be employed in the

present assay. The choice of the label will be governed by the effect of the label on the rate of hybridization and binding of the probe to mutant nucleotide sequence. It will be necessary that the label provide sufficient sensitivity to detect the amount of mutant nucleotide sequence available for hybridization.

[0163] The manner in which the label is bound to the probe will vary depending upon the nature of the label. For a radioactive label, a wide variety of techniques can be employed. Commonly employed is nick translation with a ^{32}P -dNTP or terminal phosphate hydrolysis with alkaline phosphatase followed by labeling with radioactive ^{32}P employing ^{32}P -NTP and T4 polynucleotide kinase. Alternatively, nucleotides can be synthesized where one or more of the elements present are replaced with a radioactive isotope, *e.g.*, hydrogen with tritium. If desired, complementary labeled strands can be used as probes to enhance the concentration of hybridized label.

[0164] Where other radionucleotide labels are involved, various linking groups can be employed. A terminal hydroxyl can be esterified with inorganic acids, *e.g.*, ^{32}P phosphate, or ^{14}C organic acids, or else esterified to provide linking groups to the label. Alternatively, intermediate bases may be substituted with activatable linking groups that can then be linked to a label.

[0165] Enzymes of interest as reporter groups will primarily be hydrolases, particularly esterases and glycosidases, or oxidoreductases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, and so forth. Chemiluminescers include luciferin, and 2, 3-dihydrophthalazinediones (*e.g.*, luminol).

[0166] Standard hybridization techniques for detecting a nucleic acid sequence are known in the art. The particular hybridization technique is not essential to the invention. Other hybridization techniques are described by Gall and Pardue, *Proc. Natl. Acad. Sci.* 63:378, 1969); and John, *et al.*, *Nature*, 223:582, 1969). As improvements are made in hybridization techniques they can readily be applied in the method for the invention.

[0167] The amount of labeled probe present in the hybridization solution will vary widely, depending upon the nature of the label, the amount of the labeled probe that can reasonably bind to the filter, and the stringency of the hybridization. Generally, substantial excess over stoichiometric concentrations of the probe will be employed to enhance the rate of binding of the probe to the fixed target nucleic acid.

[0168] The present invention also contemplates transgenic non-human organisms, including invertebrates, vertebrates and mammals. For purposes of the subject invention, these animals are referred to as "transgenic" when such animal has had a heterologous DNA sequence, or one or more additional DNA sequences normally endogenous to the animal (collectively referred to herein as "transgenes") chromosomally integrated into the germ cells of the animal. The transgenic animal (including its progeny) will also have the transgene integrated into the chromosomes of somatic cells.

[0169] Various methods to make the transgenic animals of the subject invention can be employed. Generally speaking, three such methods may be employed. In one such method, an embryo at the pronuclear stage (a "one cell embryo") is harvested from a female and the transgene is microinjected into the embryo, in which case the transgene will be chromosomally integrated into both the germ cells and somatic cells of the resulting mature animal. In another such method, embryonic stem cells are isolated and the transgene incorporated therein by electroporation, plasmid transfection or microinjection, followed by reintroduction of the stem cells into the embryo where they colonize and contribute to the germ line. Methods for microinjection of mammalian species are described in United States Patent No. 4,873,191. In yet another such method, embryonic cells are infected with a retrovirus containing the transgene whereby the germ cells of the embryo have the transgene chromosomally integrated therein. When the animals to be made transgenic are avian, because avian fertilized ova generally go through cell division for the first twenty hours in the oviduct, microinjection into the pronucleus of the fertilized egg is problematic due to the inaccessibility of the pronucleus. Therefore, of the methods to make transgenic animals described generally above, retrovirus infection is preferred for avian species, for example as described in U.S.

5,162,215. If microinjection is to be used with avian species, however, a published procedure by Love *et al.*, (Biotechnology, 12, Jan 1994) can be utilized whereby the embryo is obtained from a sacrificed hen approximately two and one-half hours after the laying of the previous laid egg, the transgene is microinjected into the cytoplasm of the germinal disc and the embryo is cultured in a host shell until maturity. When the animals to be made transgenic are bovine or porcine, microinjection can be hampered by the opacity of the ova thereby making the nuclei difficult to identify by traditional differential interference-contrast microscopy. To overcome this problem, the ova can first be centrifuged to segregate the pronuclei for better visualization.

[0170] The "non-human animals" of the invention include, for example, bovine, porcine, ovine and avian animals (e.g., cow, pig, sheep, chicken, turkey). The "transgenic non-human animals" of the invention are produced by introducing "transgenes" into the germ line of the non-human animal. Embryonal target cells at various developmental stages can be used to introduce transgenes. Different methods are used depending on the stage of development of the embryonal target cell. The zygote is the best target for microinjection. The use of zygotes as a target for gene transfer has a major advantage in that in most cases the injected DNA will be incorporated into the host gene before the first cleavage (Brinster *et al.*, Proc. Natl. Acad. Sci. USA 82:4438-4442, 1985). As a consequence, all cells of the transgenic non-human animal will carry the incorporated transgene. This will in general also be reflected in the efficient transmission of the transgene to offspring of the founder since 50% of the germ cells will harbor the transgene.

[0171] The term "transgenic" is used to describe an animal that includes exogenous genetic material within all of its cells. A "transgenic" animal can be produced by crossbreeding two chimeric animals that include exogenous genetic material within cells used in reproduction. Twenty-five percent of the resulting offspring will be transgenic *i.e.*, animals which include the exogenous genetic material within all of their cells in both alleles. 50% of the resulting animals will include the exogenous genetic material within one allele and 25% will include no exogenous genetic material.

[0172] In the microinjection method useful in the practice of the invention, the transgene is digested and purified free from any vector DNA *e.g.* by gel electrophoresis. It is preferred that the transgene include an operatively associated promoter which interacts with cellular proteins involved in transcription, ultimately resulting in constitutive expression. Promoters useful in this regard include those from cytomegalovirus (CMV), Moloney leukemia virus (MLV), and herpes virus, as well as those from the genes encoding metallothionein, skeletal actin, P-enolpyruvate carboxylase (PEPCK), phosphoglycerate (PGK), DHFR, and thymidine kinase. Promoters for viral long terminal repeats (LTRs) such as Rous Sarcoma Virus can also be employed. When the animals to be made transgenic are avian, preferred promoters include those for the chicken γ -globin gene, chicken lysozyme gene, and avian leukosis virus. Constructs useful in plasmid transfection of embryonic stem cells will employ additional regulatory elements well known in the art such as enhancer elements to stimulate transcription, splice acceptors, termination and polyadenylation signals, and ribosome binding sites to permit translation.

[0173] Retroviral infection can also be used to introduce transgene into a non-human animal, as described above. The developing non-human embryo can be cultured *in vitro* to the blastocyst stage. During this time, the blastomeres can be targets for retro viral infection (Jaenich, R., Proc. Natl. Acad. Sci USA 73:1260-1264, 1976). Efficient infection of the blastomeres is obtained by enzymatic treatment to remove the zona pellucida (Hogan, *et al.* (1986) in *Manipulating the Mouse Embryo*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). The viral vector system used to introduce the transgene is typically a replication-defective retro virus carrying the transgene (Jahner, *et al.*, Proc. Natl. Acad. Sci. USA 82:6927-6931, 1985; Van der Putten, *et al.*, Proc. Natl. Acad. Sci USA 82:6148-6152, 1985). Transfection is easily and efficiently obtained by culturing the blastomeres on a monolayer of virus-producing cells (Van der Putten, *supra*; Stewart, *et al.*, EMBO J. 6:383-388, 1987). Alternatively, infection can be performed at a later stage. Virus or virus-producing cells can be injected into the blastocoel (D. Jahner *et al.*, Nature 298:623-628, 1982). Most of the founders will be mosaic for the transgene since incorporation occurs only in a subset of the cells that formed the transgenic

nonhuman animal. Further, the founder may contain various retro viral insertions of the transgene at different positions in the genome that generally will segregate in the offspring. In addition, it is also possible to introduce transgenes into the germ line, albeit with low efficiency, by intrauterine retroviral infection of the mid-gestation embryo (D. Jahner *et al.*, *supra*).

[0174] A third type of target cell for transgene introduction is the embryonal stem cell (ES). ES cells are obtained from pre-implantation embryos cultured *in vitro* and fused with embryos (M. J. Evans *et al.* Nature 292:154-156, 1981; M.O. Bradley *et al.*, Nature 309:255-258, 1984; Gossler, *et al.*, Proc. Natl. Acad. Sci USA 83:9065-9069, 1986; and Robertson *et al.*, Nature 322:445-448, 1986). Transgenes can be efficiently introduced into the ES cells by DNA transfection or by retro virus-mediated transduction. Such transformed ES cells can thereafter be combined with blastocysts from a nonhuman animal. The ES cells thereafter colonize the embryo and contribute to the germ line of the resulting chimeric animal. (For review see Jaenisch, R., Science 240:1468-1474, 1988).

[0175] "Transformed" means a cell into which (or into an ancestor of which) has been introduced, by means of recombinant nucleic acid techniques, a heterologous nucleic acid molecule. "Heterologous" refers to a nucleic acid sequence that either originates from another species or is modified from either its original form or the form primarily expressed in the cell.

[0176] "Transgene" means any piece of DNA that is inserted by artifice into a cell, and becomes part of the genome of the organism (*i.e.*, either stably integrated or as a stable extrachromosomal element) that develops from that cell. Such a transgene may include a gene that is partly or entirely heterologous (*i.e.*, foreign) to the transgenic organism, or may represent a gene homologous to an endogenous gene of the organism. Included within this definition is a transgene created by the providing of an RNA sequence that is transcribed into DNA and then incorporated into the genome. The transgenes of the invention include DNA sequences which encode an invention ANKTM1-related polypeptide, and include sense, antisense, dominant negative encoding polynucleotides, which may be expressed in a transgenic non-human animal. The term

"transgenic" as used herein additionally includes any organism whose genome has been altered by *in vitro* manipulation of the early embryo or fertilized egg or by any transgenic technology to induce a specific gene knockout (*i.e.*, knockout of an invention ANKTM1-related polypeptide). The term "gene knockout" as used herein, refers to the targeted disruption of a gene *in vivo* with complete or partial loss of function that has been achieved by any transgenic technology familiar to those in the art (*e.g.*, insertion of a P-element in *Drosophila*). In one embodiment, transgenic animals having gene knockouts are those in which the target gene has been rendered nonfunctional by an insertion targeted to the gene to be rendered non-functional by homologous recombination. As used herein, the term "transgenic" includes any transgenic technology familiar to those in the art which can produce an organism carrying an introduced transgene or one in which an endogenous gene has been rendered non-functional or "knocked out."

[0177] In one embodiment, the transgene comprises DNA antisense to the coding sequence for an invention ANKTM1-related polypeptide. In another embodiment, the transgene comprises DNA encoding an antibody that is able to bind to an invention ANKTM1-related polypeptide. Where appropriate, DNA sequences that encode proteins having nociceptive activity but differ in nucleic acid sequence due to the degeneracy of the genetic code may also be used herein, as may truncated forms, allelic variants and interspecies homologues.

[0178] The invention also includes animals having heterozygous mutations in or partial inhibition of function or expression of an invention ANKTM1-related polypeptide. One of skill in the art would readily be able to determine if a particular mutation or if an antisense molecule was able to partially inhibit ANKTM1-related polypeptide. For example, *in vitro* testing may be desirable initially by comparison with wild-type (*e.g.*, comparison of northern blots to examine a decrease in expression).

[0179] After an embryo has been microinjected, colonized with transfected embryonic stem cells or infected with a retrovirus containing the transgene (except for practice of the subject invention in avian species which is addressed elsewhere herein) the embryo is implanted into the oviduct of a pseudopregnant female. The consequent progeny are

tested for incorporation of the transgene by Southern blot analysis of blood samples using transgene specific probes. PCR is particularly useful in this regard. Positive progeny (G_0) are crossbred to produce offspring (G_1), which are analyzed for transgene expression by Northern blot analysis of tissue samples. To be able to distinguish expression of like-species transgenes from expression of the animal's endogenous ANKTM1-related polypeptide gene(s), a marker gene fragment can be included in the construct in the 3' untranslated region of the transgene and the Northern probe designed to probe for the marker gene fragment. The serum levels of ANKTM1-related polypeptide can also be measured in the transgenic animal to establish appropriate expression. Expression of the nociceptive-related transgenes, thereby decreasing the ANKTM1-related polypeptide in the tissue and serum levels of the transgenic animals.

[0180] Transgenic organisms of the invention are highly useful in the production of organisms for study of tumorigenesis and in identifying agents or drugs with inhibit or modulate tumorigenesis and inheritance.

[0181] It will be recognized that the method for creating a transgenic organism include methods for inserting a transgene into, for example, an embryo of an already created transgenic organism, the organism being transgenic for a different unrelated gene or gene product.

[0182] In one embodiment the transgenic organism is an insect. An insect as used herein denotes all insect species. Typically the insect is selected from the group consisting of bristletails, springtails, mayflies, dragonflies, damselflies, grasshoppers, crickets, walkingsticks, praying-mantises, cockroaches, earwigs, termites, stoneflies, lice, thrips, bed bugs, plant bugs, damsel bugs, flower bugs, assassin bugs, ambush bugs, lace bugs, stink bugs, cicadas, treehoppers, leafhoppers, spittlebugs, planthoppers, aphids, whiteflies, beetles, scorpionflies, caddisflies, moths, skippers, butterflies, crane flies, sand flies, mosquitoes, horse flies, fruit flies louse flies, bees, wasps, and ants.

[0183] The transgenic insects of the invention can be produced by introducing into single cell embryos DNA disrupting expression of a nucleic acid encoding the wild type

ANKTM1-related polypeptide sequence. Transgenic insects can be generated by microinjection, which can produce P-element mediated germ line transformation. For transgenic insects, generally the transgene is introduced at an embryonic stage. For example, transgenic insects of the present invention can be produced by introducing into single cell embryos invention polynucleotides, either naked or contained in an appropriate vector, by microinjection, for example, which can produce insects by P-element mediated germ line transformation (see *e.g.*, Rubin *et al.*, *Science* 218:348-353 (1982)). Totipotent or pluripotent stem cells transformed by microinjection, calcium phosphate mediated precipitation, liposome fusion, retroviral infection or other means are then introduced into the embryo, and the polynucleotides are stably integrated into the genome. A transgenic embryo so transformed then develops into a mature transgenic insect in which the transgene is inherited in normal Mendelian fashion. Additional methods for producing transgenic insects can be found, for example, in O=Brochta *et al.*, *Insect Biochem. Mol. Biol.* 26:739-753 (1996) and in Louleris *et al.*, *Science* 270:2002-2005 (1995).

[0184] In one method, developing insect embryos are infected with a virus, such as a baculovirus (*e.g.*, *Autographa californica* AcNPV), containing a polynucleotide sequence of the invention, and transgenic insects produced from the infected embryo. The virus can be an occluded virus or a nonoccluded virus. A virus can be occluded by coinfection of cells with a helper virus that supplies polyhedrin gene function. The skilled artisan will understand how to construct recombinant viruses in which the polynucleotide is inserted into a nonessential region of the baculovirus genome. For example, in the AcNPV genome, nonessential regions include the p10 region (Adan *et al.*, *Virology* 444:782-793, 1982), the DA26 region (O'Reily *et al.*, *J. Gen. Virol.* 71:1029-1037, 1990), the ETL region (Crawford *et al.*, *Virology* 62:2773-2781, 1988), the *egt* region (O'Reily *et al.*, *J. Gen. Virol.* 64:1321-1328), amongst others. Significant homology exists among particular genes of different baculoviruses and therefore, one of skill in the art will understand how to insert an invention polynucleotide into similar nonessential regions of other baculoviruses. Thus, for example, a sequence encoding an invention ANKTM1-related polypeptide as described herein may be placed under control of an AcNPV promoter (*e.g.*, the polyhedrin promoter). Depending on the vector utilized, any

of a number of suitable transcription and translation elements, including constitutive, inducible and conditional promoters, enhancers, transcription terminators, etc. may be used in order to transcribe invention polynucleotides or express invention polypeptides. Alternatively, a transgene containing a nucleic acid sequence disrupting expression of an invention ANKTM1-related polypeptide may not contain a promoter as the nucleic acid sequence need not be transcribed or translated to obtain a transgenic insect having disrupted ANKTM1-related polypeptide.

[0185] Thus, the invention provides methods for producing transgenic insects having a disrupted nucleic acid sequence encoding an invention ANKTM1-related polypeptide. The methods include introducing into the genome of an insect a nucleic acid construct, including a disrupted or mutated ANKTM1-related polynucleotide sequence, and obtaining a transgenic insect having a disrupted nucleic acid sequence encoding ANKTM1-related polypeptide. The invention further provides methods for producing transgenic insects having a nucleic acid encoding ANKTM1-related polypeptide or functional fragment thereof.

[0186] Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The following examples are to be considered illustrative and thus are not limiting of the remainder of the disclosure in any way whatsoever.

EXAMPLE 1

Molecular cloning of ANKTM1

[0187] Bioinformatic searches were done as previously described (Peier et al., 2002a, supra; Peier et al., 2002b, supra). Sequence analysis was performed using the Biology Workbench at the San Diego Supercomputing Center. A 902-base pair fragment of the mouse homologue of ANKTM1 was amplified from newborn mouse DRG cDNA using the following primers:

mANK-like F2 (5'-AGTGGGGAGACTACCCTGTG) (SEQ ID NO:9) and

mANK-like R2 (5'-TTTATCATGCCCATTCCTTTGC) (SEQ ID NO:10).

[0188] From this initial sequence and subsequent hits to DNA databases, the following primers were designed to PCR-amplify full-length ANKTM1 from adult mouse trigeminal ganglia cDNA:

mANK-like start (5'TTTGGATCCGCCACCATGAAGCGCGGCTTGAGGAGG)

(SEQ ID NO:11) and

mANK-like stop(5'TTGCGGCCGCCTAAAAGTCCGGGTGGCTAATAGAAC)

(SEQ ID NO 12).

EXAMPLE 3

Expression Analysis

[0189] Overall tissue distribution of ANKTM1 was analyzed by Northern blot analysis using a probe corresponding to nucleotides 590-1492 of mouse ANKTM1 (SEQ ID NO:13). To further determine the expression pattern of ANKTM1, a rat tissue Northern blot was prepared as follows: total RNA was purified from adult tissues using Trizol (Invitrogen/Gibco Life Technologies, San Diego, CA), followed by polyA⁺ purification with OLIGOTEX™ (Qiagen, Valencia, CA) according to manufacturer protocols. Approximately 3 µg polyA⁺ RNA was electrophoresed on a 1% glyoxal gel, transferred to nylon membranes and hybridized with a ³²P-labeled probe corresponding to nucleotides 590-1492 of mouse full-length ANKTM1 (SEQ ID NO:13). Blots were hybridized 3 hours at 65°C in EXPRESSHYB™ solution (Clontech, Palo Alto, CA) and analyzed using a phosphorimager. A commercial mouse tissue northern blot (Clontech) was similarly treated.

[0190] The tissue distribution studies showed that ANKTM1 expression was not detected on a blot containing various mouse tissues, including heart, lung, skeletal muscle, and kidney (Figure 2A). However, probe of a blot containing rat tissues relevant to sensory neurons and their projections such as DRG, spinal cord, and skin detected a single mRNA species of approximately 5kb only in DRG (Figure 2B).

In situ hybridization studies

[0191] More detailed expression analysis of ANKTM1 in DRG was carried out via in situ hybridization and immunostaining. For in situ hybridization and immunostaining of DRG's, adult mice were perfused with 4% paraformaldehyde and DRG's rapidly dissected. Following post-fixation and cryo-protection in 30% sucrose in PBS, single DRG's were embedded in OCT compound, frozen in liquid nitrogen and sectioned at 10- μ m thickness. Digoxigenin-labeled ANKTM1 cRNA probes strongly hybridized to neurons in adult DRG and trigeminal ganglia (Figure 3). ANKTM1 expression was found to be restricted to a small sub-population of DRG neurons (59 of 1608 or 3.6% of the DRG neurons). To further characterize its expression in sensory neurons, tests were conducted to determine whether other DRG markers co-localized with ANKTM1. These tests showed that ANKTM1 is not expressed in heavily myelinated neurons marked by NF-150 kd (Figures 3A-3C). This finding shows that ANKTM1 is most likely expressed in the non-myelinated C- or lightly myelinated A_δ- fiber population of neurons that sense temperature and/or noxious stimuli.

EXAMPLE 3**Double in-situ hybridization studies**

[0192] The expression of ANKTM1 relative to known thermo-activated TRP channels was also studied. TRPV1 (VR1) is a well-characterized receptor for noxious heat, pH, and capsaicin. For double in-situ hybridizations, sections were hybridized with in vitro transcribed digoxigenin- or fluorescein-labeled cRNA probes (Roche, Basel, Switzerland) corresponding to nucleotides 590-1492 of mANKTM1, and nucleotides 1410-1980 of mTRPM8 (NM_029310) (SEQ ID NO:14). We used two cRNA probes corresponding to bases 1516-2065 or 1516-2482 of TRPV1 sequence (AF029310). Both probes showed consistent patterns of hybridization. Peroxidase-conjugated anti-digoxigenin-POD (1:500) and alkaline phosphatase-conjugated anti-fluorescein (1:2000) antibodies (Roche) were used to detect hybridized cRNA probes and visualized using tyramide signal amplification (TSA; NEN) and fast-red detection (Roche) systems, respectively. The immunostaining experiments followed hybridization of sections with digoxigenin-labeled

cRNA probes and TSA fluorescent detection. Anti-NF150kd (1:1000; Chemicon, Temecula, CA) and anti-CGRP (1:100; Biogenesis,) primary antibodies and anti-rabbit Cy3 (1:200, Jackson ImmunoResearch,) secondary antibodies were used.

[0193] The results of these experiments utilizing ANKTM1 and TRPV1 probes (Figures 3G-3I) revealed that 97%, or 100 out of 103, of ANKTM1-positive neurons also express TRPV1, while 30% (or 100 out of 336) of TRPV1-positive neurons express ANKTM1. This expression pattern differs from that of TRPM8, the cold and menthol receptor, which is not coexpressed with CGRP and TRPV1 in DRG neurons. Since ANKTM1 expression overlaps with these two markers, coexpression of ANKTM1 and TRPM8 would not be expected. However, these double in situ hybridizations revealed no overlap of expression between ANKTM1 and TRPM8 (n=113 for ANKTM1 and 137 for TRPM8) (Figures 3J-3L). Taken together, these results indicate that ANKTM1 is expressed in a sub-population of nociceptive/thermoceptive neurons that coincides with noxious heat-activated TRPV1 receptor, but not with the cool/cold-activated TRPM8 receptor.

[0194] In accordance with this observation, it was further discovered that ANKTM1 is expressed in Calcitonin gene-related peptide (CGRP)- and in Substance P (SP)-positive neurons (Figures 3D-3F). 97% or 69 out of 71 of these ANKTM1-positive neurons were CGRP-positive. Both CGRP and SP are secreted inflammatory peptides expressed in a subset of nociceptive neurons (Scott, 1992).

EXAMPLE 4

CHO Cell Expression System

[0195] Nevertheless, since ANKTM1 bears similarities to TRP-like channels expressed in sensory neurons, tests were conducted to determine activation of ANKTM1 by various sensory stimuli. To this end, a series of tests were conducted using full-length murine ANKTM1 stably transfected in Chinese Hamster Ovary (CHO) cells containing FRT sites (CHO-K1/ FRT) under control of a tetracycline (Tet)-inducible promoter via Flp recombinase mediated recombination.

[0196] CHO-K1/FRT cells were stably transfected with full-length murine ANKTM1 in the pcDNA5/FRT vector using Lipofectamine (Invitrogen) according to the manufacturer's protocol. Transfected cells were selected in growth medium containing Hygromycin (200 µg/mL). Northern blot analysis identified stable clones expressing ANKTM1 mRNA. The generation of stable murine TRPM8-expressing CHO-K1/FRT cells has been previously described (Peier et al, 2002). ANKTM1-expressing stable CHO-K1/FRT lines appeared unhealthy (membrane blebbing, cytoplasmic granulations) in culture, and after several passages, a loss of ANKTM1 expression was observed. Previous investigators have also claimed difficulty generating stable cell lines expressing human ANKTM1.

[0197] To circumvent this problem, cell lines were generated in which ANKTM1 expression could be controlled. Tetracycline-inducible CHO-K1/FRT cells lines were generated by transfecting the CHO Flp-In host cell line with pcDNA6/TR according to manufacturer's instructions (Invitrogen, San Diego, CA) and selected in 5 µg/mL Blasticidin. Clones stably expressing the tetracycline repressor identified by blasticidin resistance were transiently transfected with a control plasmid containing the CAT gene and selected based on high levels of CAT expression after treatment with tetracycline. The gene expression vector, pcDNA5FRT/TO containing full-length murine ANKTM1 and the Flp recombinase expression plasmid pOG44 were co-transfected into the tetracycline-inducible CHO-K1/FRT cell line and selected via Hygromycin resistance (200 µg/ml). Induction of ANKTM1 was accomplished by treating CHO cells with 1-2 µg/mL tetracycline (Tet) 5-24 hours before experiments.

[0198] Stable clones expressing ANKTM1 were identified by Northern blot analysis. Northern blot analysis of control and Tet-treated ANKTM1-inducible CHO cells showed high levels of ANKTM1 expression. A low but not absent level in the absence of Tet was also present in these cells. Therefore, CHO cells that stably expressed the Tet-repressor were used as controls in subsequent experiments. For some of the experiments, cells were maintained in culture medium supplemented with 5 µm ruthenium red (RR, Fluka), which blocks ANKTM1 activity. This was done to overcome the slight leaky expression

of ANKTM1 in the Tet-system. Cells cultured in RR looked healthier and a higher percentage of cells responded to cold compared to non-RR treated cells.

[0199] As shown in Figure 4A, when buffer is cooled, $[Ca^{2+}]_i$ increases rapidly in ANKTM1-expressing CHO cells, but not in untransfected CHO cells. Average activation temperature (measured at the inlet of the cell chamber by a miniature thermocouple) is approximately 17°C. Cooling in the presence of 5µM ruthenium red and in the absence of extracellular Ca^{2+} eliminates cold-evoked responses. The threshold of activation was not affected.

EXAMPLE 5

Intracellular calcium imaging experiments

[0200] Calcium imaging experiments were performed essentially as described (Peier et al., 2002a, supra). Briefly, cells were plated on glass coverslips 24-48 hours prior to imaging experiments. Cells were washed in HEPES buffered saline solution (2 mM Ca^{2+}), loaded with Fura-2 acetoxymethyl ester (5 mM), 1.5 mM pluronic acid (Molecular Probes, Eugene, OR) and incubated for 1 hour at room temperature in the dark. Coverslips were placed in laminar flow chamber (Warner Instrument Corp., Hamden, CN) and perfused with HEPES buffered saline (2mM Ca^{2+}) flowing through small-diameter tubing at the inlet of the chamber. Perfusion rate was approximately 2 ml/min and was stopped and started via a solenoid switch (Warner Instrument Corp., Hamden, CN). Perfusate temperature was controlled by a Peltier device and monitored via miniature thermocouple at the inlet of the chamber. Heated (up to 52°C), chilled (down to 9°C, the limit of our system), and room temperature buffer was delivered through the same application tubing at the inlet of the chamber. Alternatively, in experiments where the compounds menthol (Sigma, St. Louis, MO), capsaicin (Fluka, Buchs, Switzerland) and Icilin (Tocris Woodson,) were applied, cells were plated on 24-well tissue culture plates and loaded with Fura-2. Compounds were delivered with a 3cc syringe during a period of ten seconds.

[0201] Images of Fura-2 loaded cells with the excitation wavelength alternating between 340 nm and 380 nm were captured with a cooled LCD camera. The ratio of fluorescence intensity of the two wavelengths of groups of 30-50 cells in each experiment was analyzed using MetaFluor (Universal Imaging Corp). Experiments were performed in triplicate. Unless otherwise indicated, graphs represent averaged responses of 20-40 cells from representative experiments. For direct comparison of results from different experiments, values were normalized to baseline of the ratio 340/380. For ANKTM1 and TRPM8 threshold experiments, the threshold temperature of activation of 60-100 cells each from three replicate experiments was analyzed. Threshold of activation was defined as 20% above baseline. Hanks Balanced Salt Solution (HBSS) and HEPES buffers were obtained from GibcoBRL. The results of these experiments are shown in Figure 5 herein.

EXAMPLE 6

Electrophysiology

[0202] Cells were plated onto poly-D-lysine-coated coverslips for recording purposes, and recordings were undertaken 18-24 hr later. Experiments were carried out at room temperature using whole-cell voltage clamp technique, with an Axopatch 2B amplifier filtered at 5 kHz and pClamp suite of software (Axon Instruments). Series-resistance compensation was 80% for all experiments, using 2-5 M Ω fire-polished pipettes. Recording solutions were as follows: pipette solution for all experiments was [(mM) CsCl, 140; BAPTA, 10; HEPES, 10; MgATP, 2; titrated to pH7.4 with CsOH. The external solution in the recording chamber was kept at 32°C and consisted of [(mM): CholineCl, 100; NaCl, 40; HEPES, 10; CaCl₂, 2; MgCl₂ 1; titrated to pH 7.4 with NaOH]. In some studies the above solution was used with 140mM NaCl and no Choline. In monovalent permeability studies, equimolar KCl or CsCl replaced NaCl (40mM). In divalent permeability studies, the solutions either contained 1 mM of test ion and (mM) CholineCl, 100; NaCl, 40; Hepes, 10; sucrose, 80 or 30mM test ion in the above solution without sucrose. The current-voltage relationships were determined using a 2 second ramp from -100 to +80mV.

[0203] Permeability ratios relative to Na^+ , were calculated for monovalent cations as follows: $P_X/P_{\text{Na}} = E_{\text{shift}} = \{RT/F\} \log (P_X/P_{\text{Na}} [X]_o/[Na]_o)$, where F is Faraday's constant, R is the universal gas constant, and T is absolute temperature. Permeability ratios relative to Na^+ , for divalent cations were calculated as follows: $E_{\text{shift}} = \{RT/F\} \log \{[Na]_o + 4B' [Ca]_{o(2)}\} / \{[Na]_o + 4B' [Ca]_{o(1)}\}$, where $B' = P'_{\text{Ca}} / P_{\text{Na}}$ and $P'_{\text{Ca}} = P_{\text{Ca}} / (1 + e^{EF/RT})$ and $[Ca]_{o(1)}$ and $[Ca]_{o(2)}$ refer to the two different concentrations of the divalent ion tested.

[0204] ANKTM1 and TRPM8 were cloned into the pOX expression vector for expression in *Xenopus* oocytes (Jegla and Salkoff, 1997). Capped cRNAs were prepared by run-off transcription using the T3 mMessage mMachine kit (Ambion) and cleaned prior to injection using QIAQUICK™ columns (Qiagen). Mature oocytes were isolated enzymatically using known methodology. Oocytes were injected with 5 ng to 25 ng of cRNA in a 50 nl volume and incubated 2-5 days prior to recording at 18°C in ND96 (96mM NaCl, 2mM KCl, 1.8 mM CaCl, 1 mM MgCl, 5 mM Hepes, 2.5 mM Na-pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, pH7.5). Recordings were made using standard two-electrode voltage clamp techniques with 3 M KCl electrodes (0.3-0.5 MΩ). The recording solution consisted of 96 mM NaCl, 4 mM KCl, 1 mM MgCl, 100 µM CaCl and 5 mM Hepes, pH 7.5. Temperature control was achieved with a combination of a peltier-cooled stage and constant perfusion of cooled/heated solution. Temperatures were monitored using a miniature thermocouple placed adjacent to the oocyte in the recording chamber. Currents and temperatures were recorded using a TEV-200 amplifier (Dagan Instruments, Minneapolis, MN), an HCC-100A temperature controller (Dagan Instruments), and pCLAMP8™ software suite (Axon Instruments, Union City, CA). The oocytes were held at -70mV during the recordings.

[0205] Figures 7A-D are a series of graphs summarizing the results of these experiments. Figures 7A and 7C show, respectively, inward currents recorded in response to first and second cold steps from *Xenopus* oocytes expressing ANKTM1; while Figures 7B and 7D show, respectively, inward currents recorded in response to cold

steps from *Xenopus* oocytes expressing TRPM8. By comparison to TRPM8, ANKTM1 current responses were markedly reduced in the second cold step.

[0206] It will be apparent to those skilled in the art that various modifications and variations can be made to the compounds and processes of this invention. Thus, it is intended that the present invention cover such modifications and variations, provided they come within the scope of the appended claims. Accordingly, the invention is limited only by the following claims.

WHAT IS CLAIMED IS:

1. An isolated nucleic acid sequence encoding an ANKTM1-related polypeptide, characterized as
 - encoding a temperature/pain sensitive non-selective cation channel protein that is activated by temperature below 20 °C;
 - being expressed in Calcitonin gene-related peptide- and substance P-positive neurons; and
 - comprising more than five ankyrin domains and a six transmembrane domain.
2. The isolated nucleic acid sequence of claim 1 encoding a ANKTM1-related polypeptide comprising an amino acid sequence selected from SEQ ID NO:1, SEQ ID NO:2, and conservative variations thereof.
3. The isolated nucleic acid sequence of claim 2, wherein the ANKTM1-related polypeptide comprises the amino acid sequence of SEQ ID NO:1, or a conservative variation thereof.
4. The isolated nucleic acid sequence of claim 2 wherein the ANKTM1-related polypeptide comprises the amino acid sequence of SEQ ID NO:2, or a conservative variation thereof.
5. A method for identifying an agent that modulates nociceptive response, the method comprising:
 - (a) contacting an organism containing an ANKTM1-related polypeptide encoded by the sequence set forth in claim 1 with an agent suspected of having nociceptive pain modulating activity under conditions that allow the agent and the polypeptide to interact;
 - (b) measuring a nociceptive response to administration of a nociceptive stimulus to the organism;

- (c) and comparing the nociceptive response to a nociceptive response to the stimulus in the organism when not administered the agent, wherein a change in the nociceptive response indicates an agent that modulates the nociceptive response.
6. The method for claim 5, wherein the agent is selected from the group consisting of a peptide, a peptidomimetic, a chemical, and a nucleic acid sequence.
 7. The method for claim 5, wherein modulation of the nociceptive response is a decrease in pain and the change in the nociceptive response indicates that the agent decreases nociceptive pain.
 8. The method for claim 5, wherein the organism is a non-human organism.
 9. The method for claim 8, wherein the non-human organism is a mammalian cell.
 10. The method for claim 9, wherein stimulus is noxious cold and the response comprises a rise in $[Ca^{2+}]_i$ or $[Mg^{2+}]_i$ in the cell.
 11. The method for claim 5, wherein the organism is a vertebrate.
 12. The method for claim 11, wherein the vertebrate is a murine species.
 13. The method for claim 11, wherein the organism is a mammal.
 14. The method for claim 5, wherein the organism is a human.

15. A method for modulating nociceptive pain in an organism, the method comprising contacting a sentient organism containing a polypeptide sequence comprising an amino acid sequence selected from SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:4, and conservative variations thereof, with an effective amount of an agent that modulates operation of the polypeptide under conditions that allow the agent and the polypeptide to interact, thereby modulating nociceptive pain in the organism.
16. The method for claim 15, wherein the polypeptide contains an ion channel and the agent modifies operation of the ion channel.
17. The method for claim 15, wherein the modulating is a decrease in the nociceptive pain.
18. The method for claim 15, wherein the agent is selected from the group consisting of a peptide, a peptidomimetic, a chemical, and a nucleic acid sequence.
19. The method for claim 15, wherein the organism is a non-human organism.
20. The method for claim 15, wherein the organism is an vertebrate.
21. The method for claim 15, wherein the vertebrate is a murine species.
22. The method for claim 15, wherein the organism is a human.
23. The method for claim 15, wherein the organism is a transgenic organism.
24. The method for claim 17, wherein the transgenic organism is a mammal.

25. A method for identifying an agent that modulates nociceptive pain, the method comprising:

- (a) contacting an organism containing the polynucleotide sequence of claim 1 with a candidate agent under conditions that allow the agent and the polynucleotide to interact;
- (b) measuring a nociceptive response to administration of a nociceptive stimulus to the organism; and
- (c) comparing the nociceptive response to a nociceptive response in the organism when not administered the nociceptive stimulus,

wherein a change in the nociceptive response indicates the agent modulates nociceptive pain.

26. The method for claim 25, wherein the agent is selected from the group consisting of a peptide, a peptidomimetic, a chemical, and a nucleic acid sequence.

27. The method for claim 25, wherein the organism is a non-human organism.

28. The method for claim 25, wherein the organism is an invertebrate.

29. The method for claim 25, wherein the invertebrate is a *Drosophila* sp.

30. The method for claim 25, wherein the organism is a transgenic organism.

31. The method for claim 25, wherein the organism is a mammal.

32. A method for reducing nociceptive pain in an organism, the method comprising contacting an organism containing the polynucleotide sequence of claim 1 with an effective amount of an agent that blocks function of the polynucleotide sequence under conditions that allow the agent and the polynucleotide to interact, thereby reducing nociceptive pain in the organism.

33. The method for claim 32, wherein the agent is selected from the group consisting of a peptide, a peptidomimetic, a chemical, and a nucleic acid sequence.
34. The method for claim 32, wherein the organism is a non-human organism.
35. The method for claim 32, wherein the organism is an invertebrate.
36. The method for claim 32, wherein the invertebrate is murine
37. The method for claim 32, wherein the organism is a transgenic organism.
38. The method for claim 32, wherein the organism is a mammal.
39. An isolated polypeptide comprising an amino acid sequence selected from SEQ ID NO:1, SEQ ID NO:2 and conservative variations thereof.

ABSTRACT

The methods and compositions of the invention are based on a method for measuring nociceptive responses in vertebrates, including humans and other mammals utilizing a newly discovered thermoreceptor belonging to the Transient Receptor Potential (TRP) family of non-selective cation channels that participates in thermosensation and pain.. This receptor, designated ANKTM1, is associated with nociceptive pain, such as hyperalgesia. Accordingly, the invention provides isolated polypeptides and polynucleotides associated with nociception as well as methods for identifying or screening agents that modulate nociception.

Figure 2, Story et al.

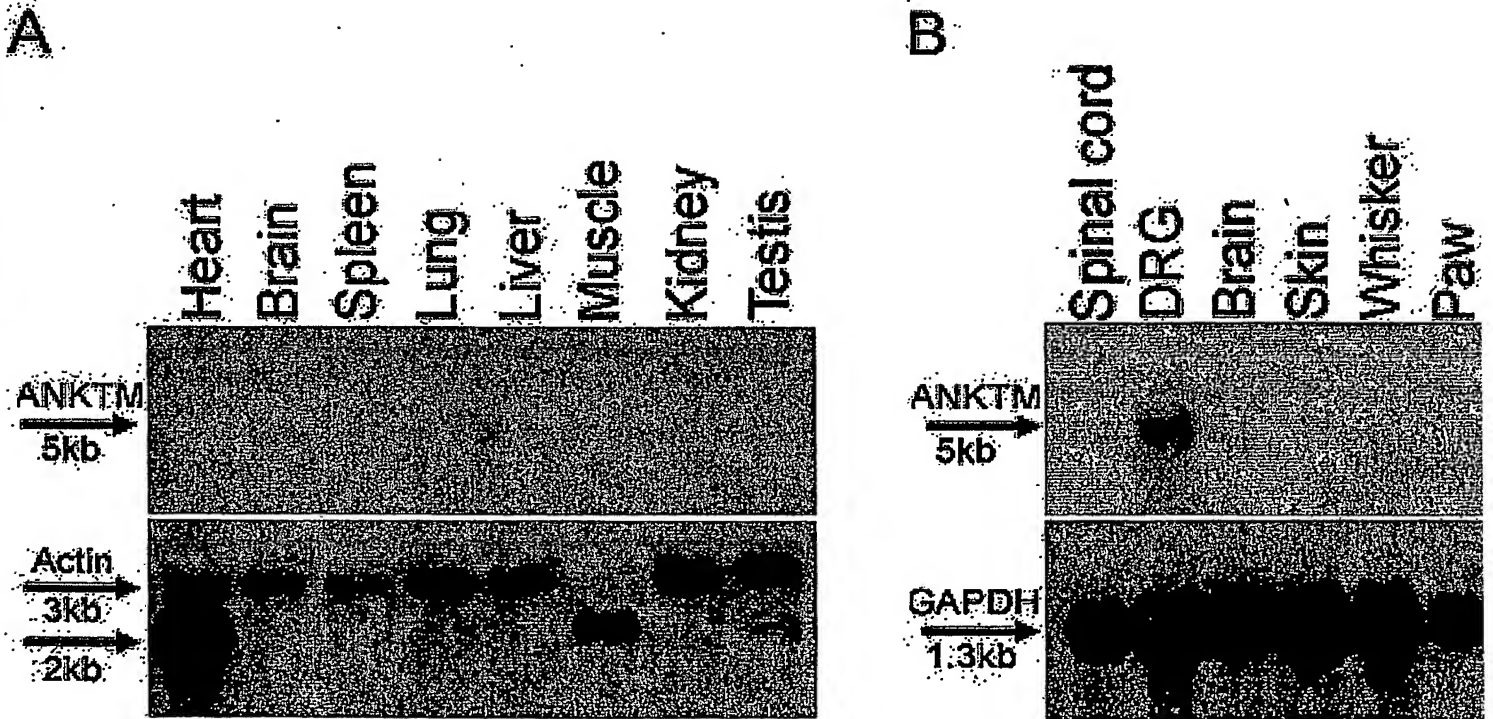


Figure 3, Cory et al.

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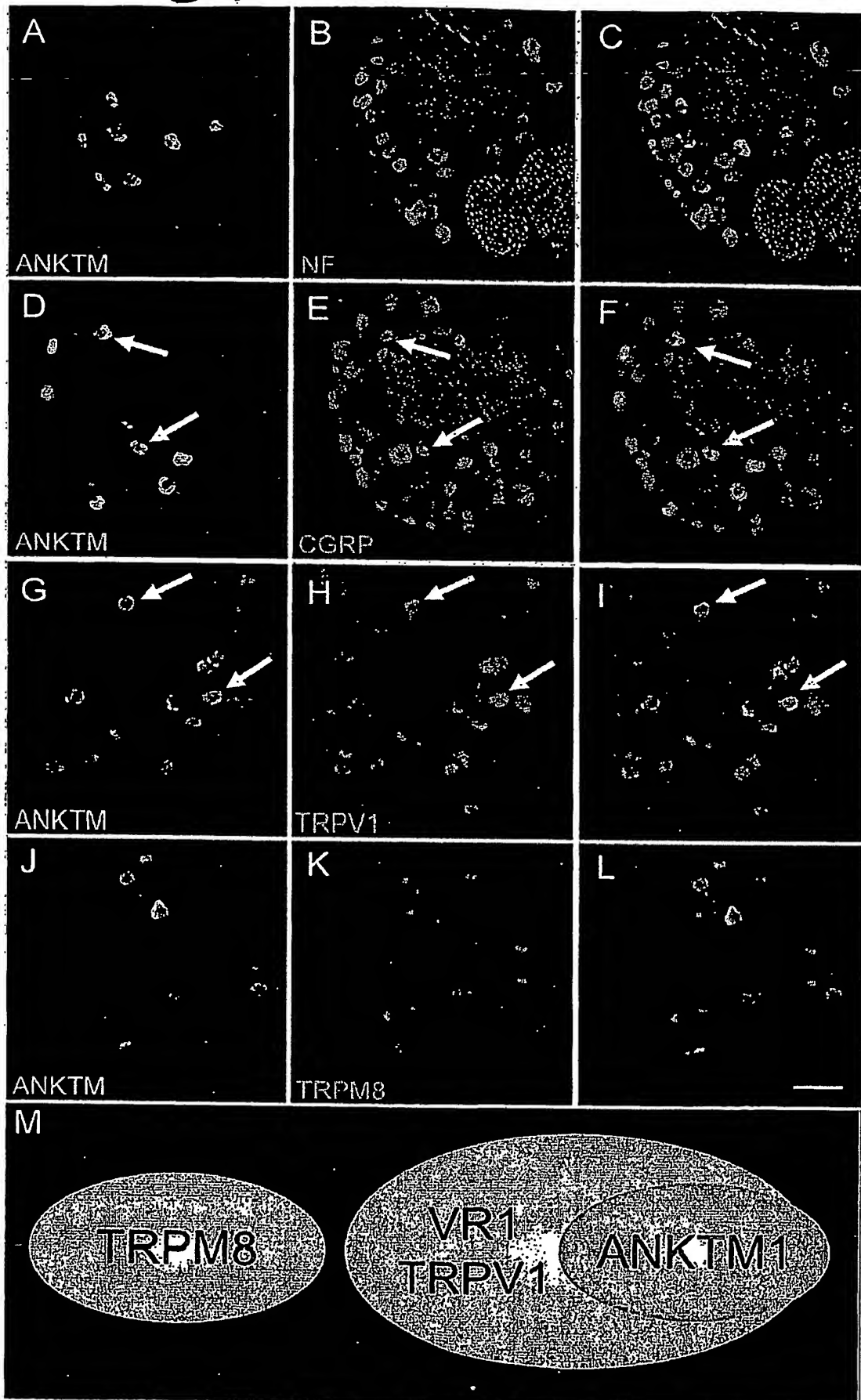
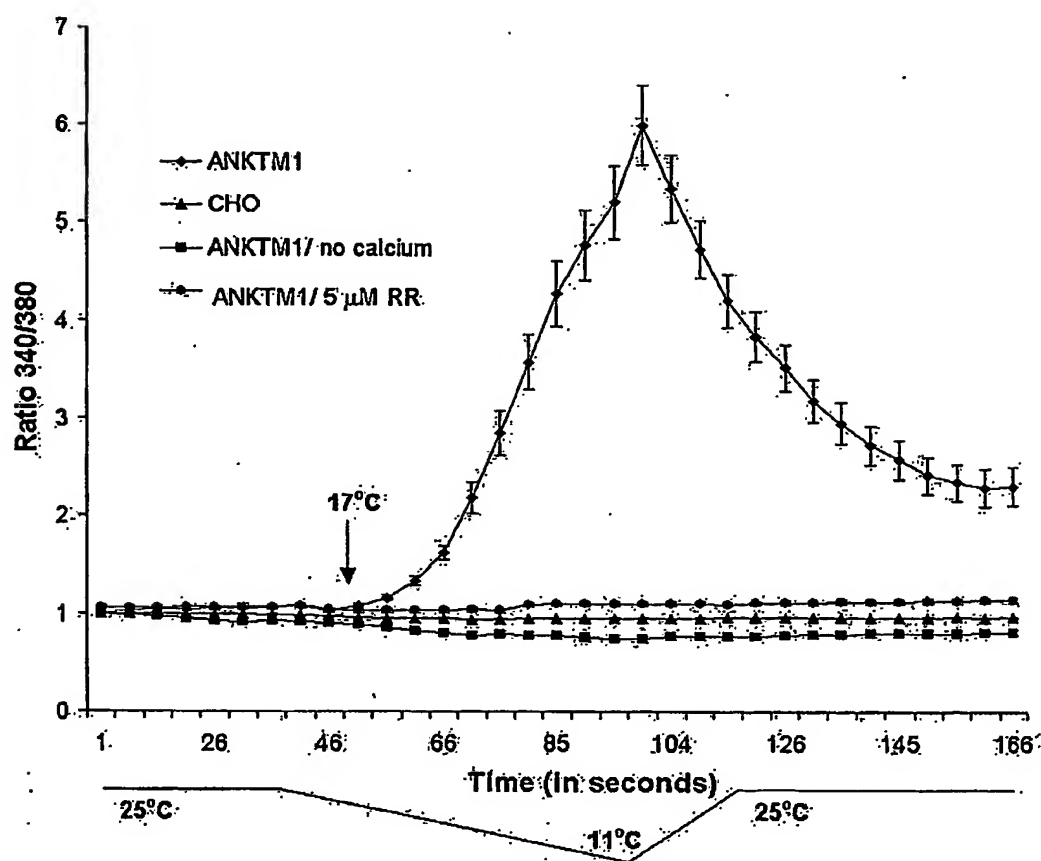


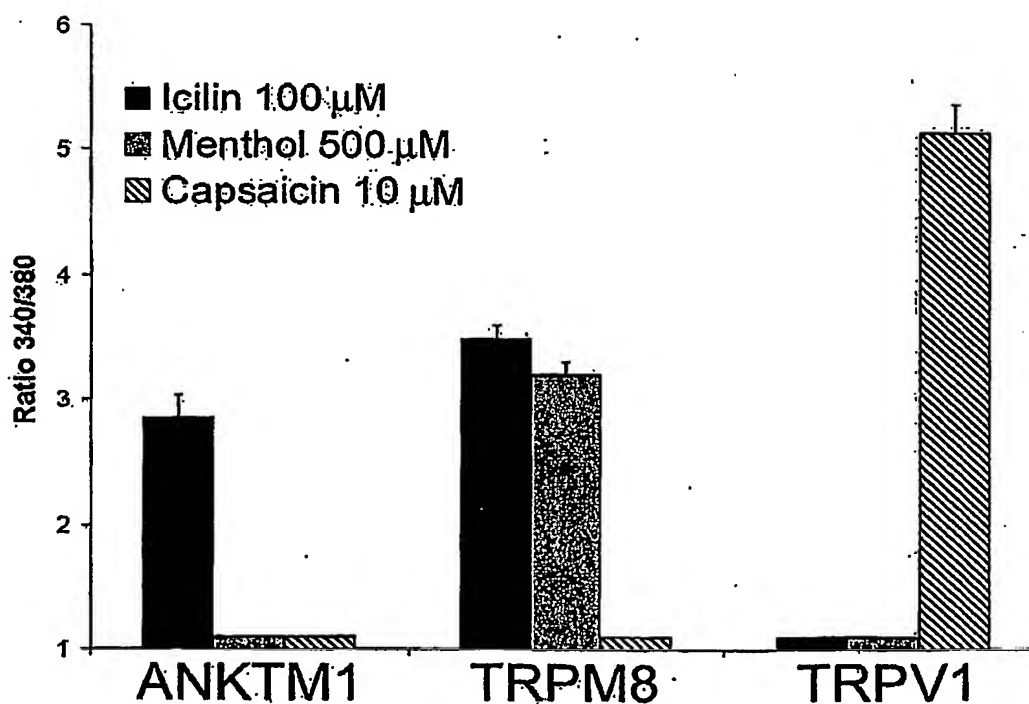
Figure 4, Story et al.

6043540.121802

A



B



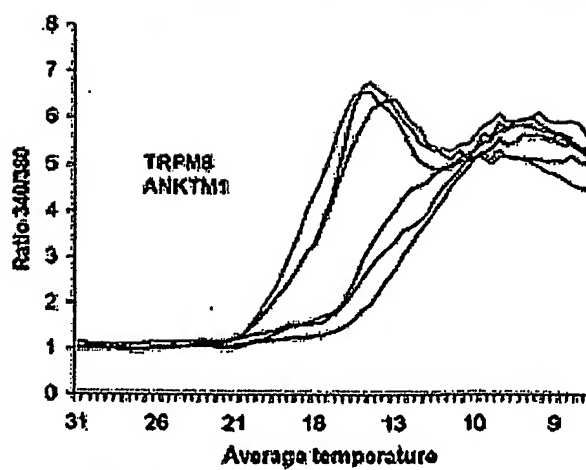
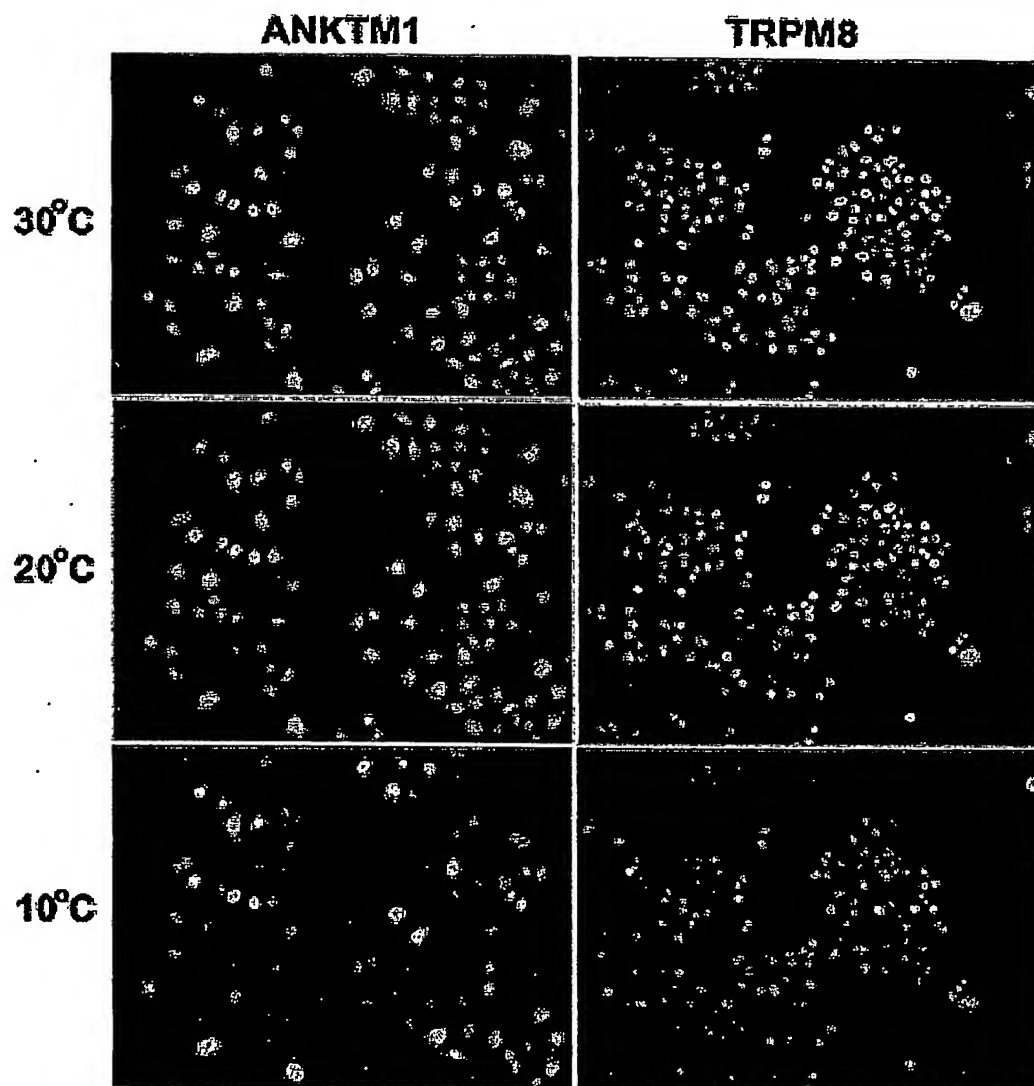
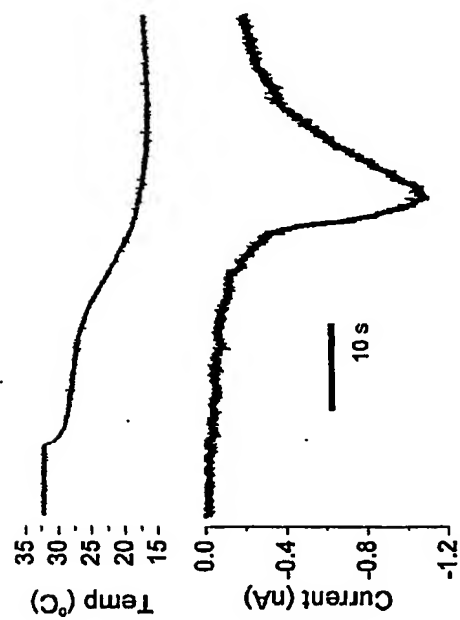
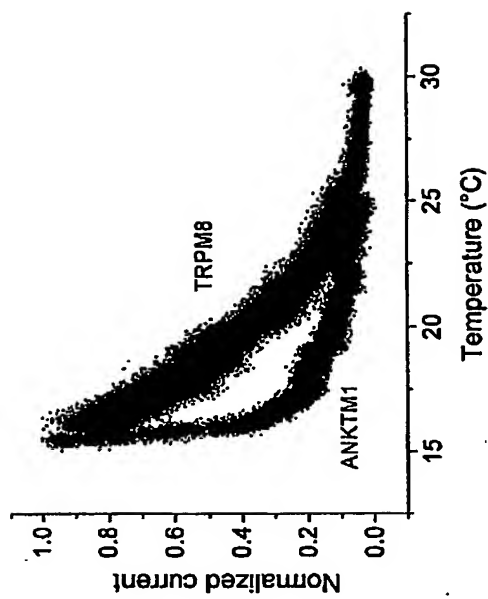


Figure 6, Story et al.

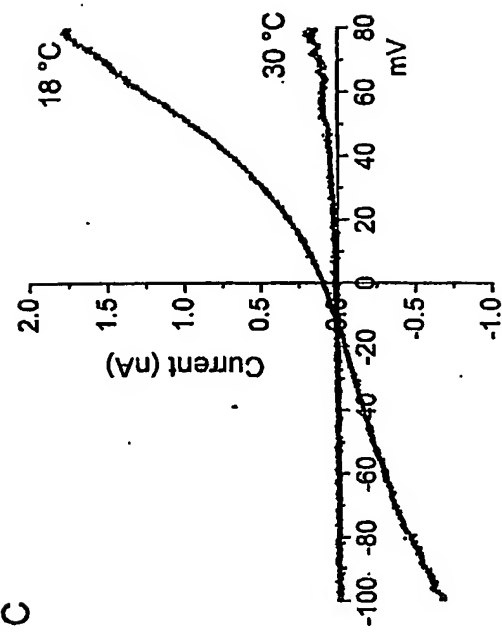
A



B



C



D

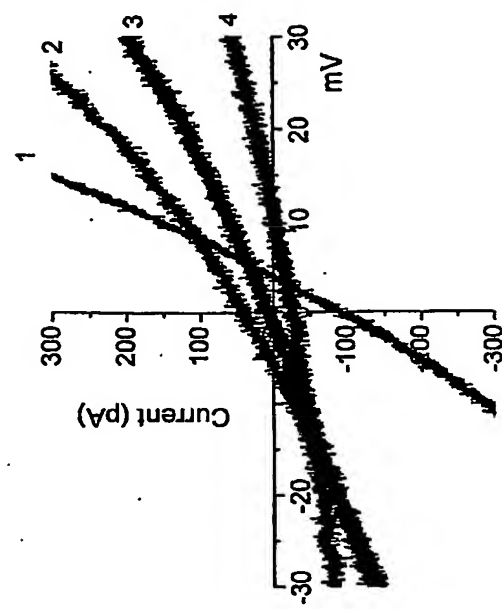


Figure 7, Story et al.

SCANNED, # _____

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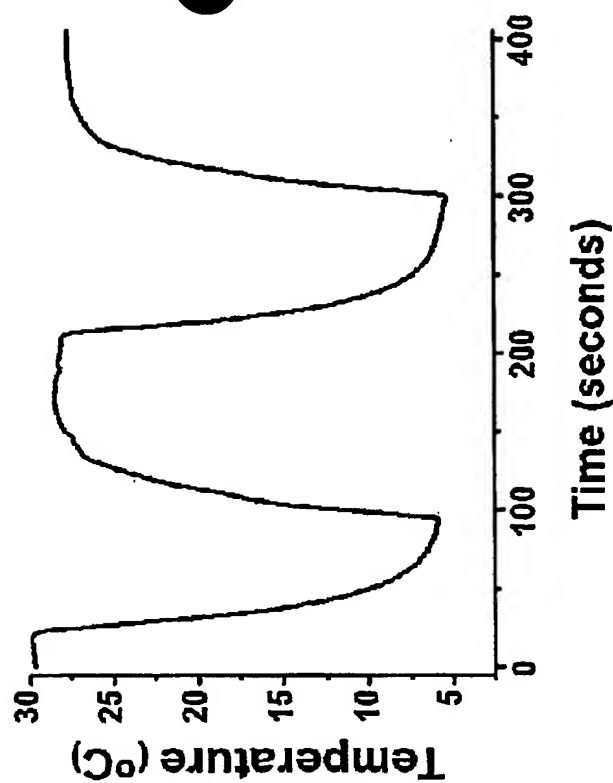
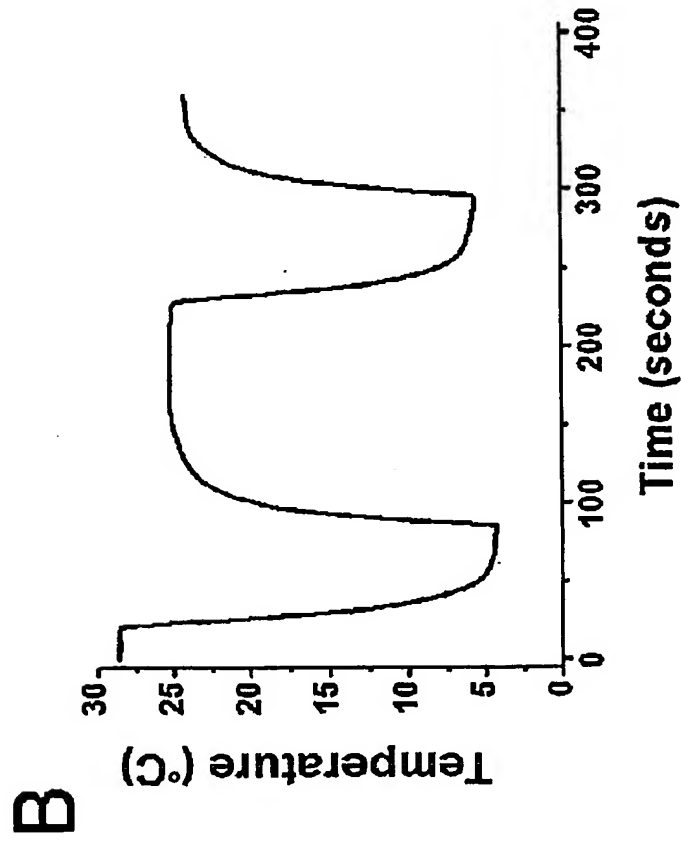
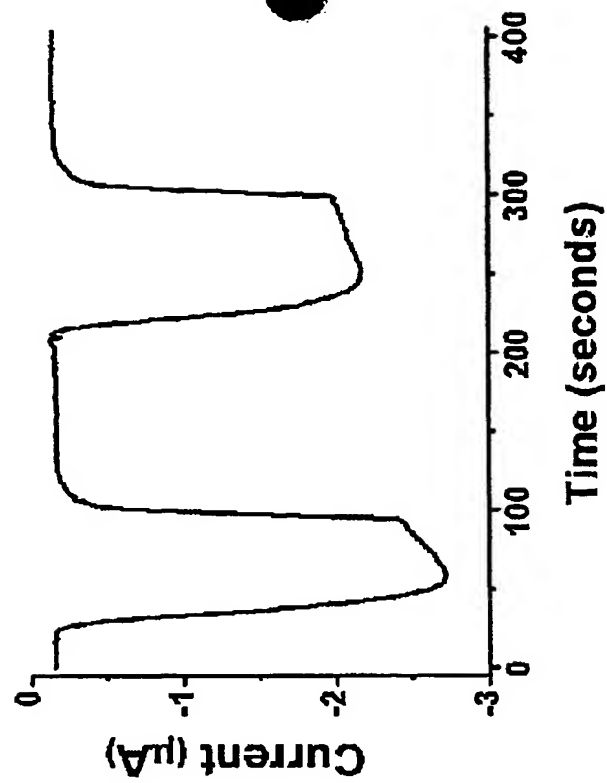
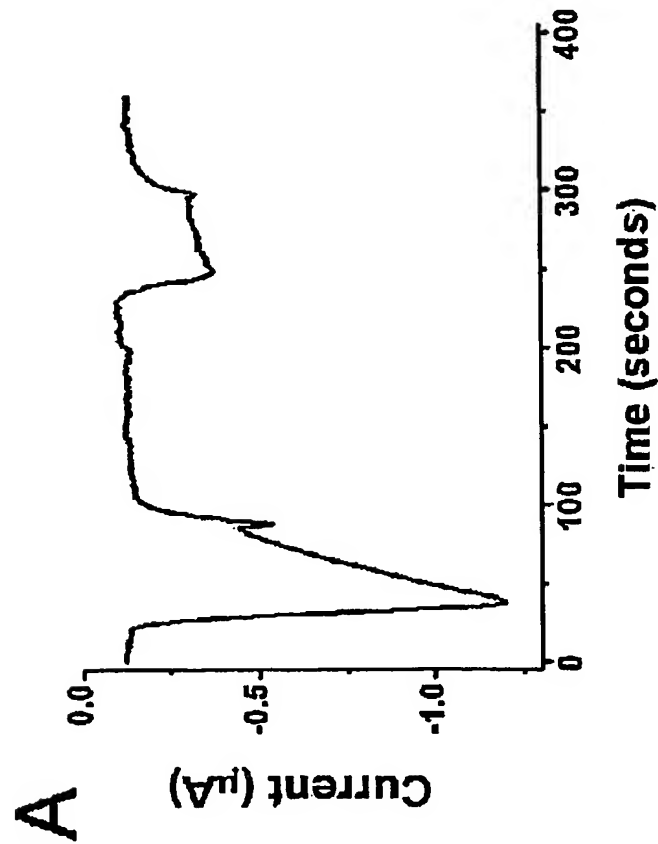


FIGURE 8A

mANKTM1 cds, 3.378 kb:

ATGAAGCGCGGCTTGAGGAGGATTCTGCTCCCGGAGGAAAGGAAGGAGGTC
CAGGGCGTTGTCTATCGCGGCGTCGGGGAAGACATGGACTGCTCCAAGGAAT
CCTTTAAGGTGGACATTGAAGGAGATATGTGTAGATTAGAAGACTTCATCAA
GAACCGAAGAAAACTAAGCAAATATGAGGATGAAAATCTCTGTCTCTGCAT
CACGCAGCAGCAGAAGGTCAAGTTGAACTGATGGAAGTATCATCAATGGTT
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GAGCAAATCCAAACCTCCGAAATAGAAACATGATGTCACCCCTTCACATAGC
TGTGCATGGCATGTACAACGAAGTGATCAAGGTGTTGACTGAGCACAAGGCC
ACTAACATCAATTTAGAAGGAGAGAATGGGAACACGGCTTTGATGTCCACGT
GTGCCAAAGACAACAGTGAAGCTTTGCAAATTTTGTTAGAAAAAGGAGCTAA
GCTGTGTAAATCAAATAAGTGGGGAGACTACCTGTGCACCAGGCAGCATT
TCAGGTGCCAAAAAATGCATGGAATTAATCTTAGCATATGGTGAAAAGAACG
GCTACAGCAGGGAGACTCACATTAATTTTGTGAATCACAAGAAAGCCAGCCC
TCTCCACCTAGCAGTTCAAAGCGGAGACTTGGACATGATTAAGATGTGCCTG
GACAACGGTGCACACATCGACATGATGGAGAATGCCAAATGCATGGCCCTCC
ATTTTGCTGCAACCCAGGGAGCCACTGACATCGTTAAGCTCATGATCTCATCC
TATACCGGAAGTAGTGATATTGTGAATGCAGTTGATGGCAATCAGGAGACCC
TGCTTCACAGAGCCTCGTTATTTGATCACCATGACCTGGCAGAATACCTAATA
TCAGTGGGAGCAGACATCAACAGCACTGATTCTGAAGGACGCTCTCCACTTA
TTTAGCAACAGCTTCTGCATCCTGGAACATTGTGAATTTGCTCCTCTGTAAA
GGTGCCAAAGTAGACATAAAAGATCATCTTGGACGTAACCTTTTGCATTTGAC
TGTGCAGCAGCCTTATGGACTAAGAAATTTGCGGCCTGAGTTTATGCAGATG
CAACACATCAAAGAGCTGGTGATGGATGAAGACAATGATGGATGCACACCTC
TCCATTATGCCTGTAGGCAGGGGGTTCTGTCTCTGTAAATAACCTCCTTGGC
TTCAATGTGTCCATTCATAGCAAAAGTAAAGATAAGAAGTCGCCCCCTGCATT
TGCAGCCAGTTATGGGCGCATCAATACATGTCAGAGACTTCTGCAAGACATA
AGTGATACGAGGCTTTTGAATGAAGGGGATCTCCATGGGATGACCCCTCTCC
ACCTGGCAGCAAAAAATGGGCATGATAAAGTCGTTCAACTCCTTCTGAAGAA
AGGGGCCTTATTTCTCAGTGACCACAATGGCTGGACTGCTTTGCATCACGCCT
CCATGGGTGGGTACACTCAGACCATGAAGGTCATTCTTGATACTAACTTGAA
ATGCACAGACCGACTAGATGAAGAAGGGAACACAGCACTCCACTTTGCAGCA
CGGGAAGGCCATGCCAAGGCTGTTGCAATGCTTTTGAGCTACAATGCTGACA
TCCTCCTGAACAAGAAGCAAGCTTCCTTTCTGCATATTGCCCTGCACAATAAG
CGCAAGGAAGTGGTTCTCACAACCATCAGAAATAAAAGATGGGATGAGTGTC
TTCAAGTTTTCACCTCATAATTCTCCAAGCAATCGATGTCCAATCATGGAGATG
GTAGAATACCTCCCCGAGTGCATGAAAGTTCTTTTAGATTTCTGCATGATACC
TTCCACAGAAGACAAGTCCTGTCAAGACTACCATATTGAGTATAATTTCAAGT
ATCTCCAATGCCCATATCCATGACCAAAAAAGTAGCACCTACCCAGGATGT
GGTATATGAGCCTCTTACAATCCTCAATGTCATGGTCCAACATAACCGCATAG

FIGURE 8B

AACTCCTCAACCACCCTGTGTGTAGGGAGTACTTACTCATGAAATGGTGTGCC
TATGGATTGAGAGCCCATATGATGAACCTAGGATCTTATTGTCCTGGTCTCAT
ACCCATGACCCTTCTTGTTGTCAAAATACAGCCTGGAATGGCCTTCAATTCTA
CTGGAATAATCAATGGAAGTAGTAGTACTCATGAGGAAAGAATAGACACTCT
GAATTCATTTCCAATAAAAAATATGTATGATTCTAGTTTTTTTTATCAAGTATATT
TGGATATTGCAAAGAAGTGATCCAAATTTTCCAACAGAAAAGGAATTACTTC
CTGGATTACAACAATGCTCTGGAATGGGTTATCTATACAACTAGTATCATCTT
CGTGTGCCCCTTGTTCCCTCAACATCCCAGCGTATATGCAGTGGCAATGTGGAG
CAATAGCGATATTCTTCTACTGGATGAACTTCCTACTGTATCTTCAAAGGTTT
GAGAACTGTGGAATTTTCATTGTTATGTTGGAGGTGATTTTTAAACATTGCT
GAGATCGACCGGAGTGTTTATCTTCCTCCTACTGGCTTTTGGCCTCAGCTTTTA
TGTTCTCCTGAATTTCCAAGATGCCTTCAGCACCCCATTGCTTTCCTTAATCCA
GACATTCAGTATGATGCTAGGAGACATCAATTATCGAGATGCCTTCCTAGAA
CCATTGTTTAGAAATGAGTTGGCATAACCCAGTCCTGACCTTTGGGCAGCTTAT
TGCCTTCACAATGTTTGTCCCAATTGTTCTCATGAACTTACTGATTGGCTTGGC
GGTTGGGGACATTGCTGAGGTCCAGAAGCATGCGTCATTGAAGAGGATTGCT
ATGCAGGTGGAACCTTCATACCAACTTAGAAAAAAGCTGCCACTCTGGTACT
TACGCAAAGTGGATCAGAGGTCCACCATCGTGTATCCAAATAGACCCAGGCA
CGGCAGGATGCTACGGTTTTTTTCACTTTCTTAATATGCAAGAAACACGAC
AAGAAGTACCAAACATTGACACATGCTTGGAATGGAAATATTGAAACAGAA
ATATCGGCTGAAGGACCTCACTTCCCTCTTGGAAGAGCAGCATGAGCTCATC
AAACTCATCATCCAGAAGATGGAGATCATCTCAGAGACAGAAGATGAAGATA
ACCATTGCTCTTTCCAAGACAGGTTCAAGAAGGAGAGGCTGGAACAGATGCA
CAGCAAGTGGAATTTTGTCTTAAACGCAGTTAAGACTAAAACACATTGTTCTA
TTAGCCACCCGGACTTTTAG

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